Universidade de Aveiro Departamento de Química
2008

CLÁUDIA SOFIA CORDEIRO NUNES

EFEITO DO PROCESSAMENTO NAS CARACTERÍSTICAS FÍSICO-QUÍMICAS DA AMEIXA D'ELVAS

Alterações ao Nível dos Polissacarídeos das Paredes Celulares, Enzimas, Aroma e Textura

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Bioquímica, realizada sob a orientação científica do Doutor Manuel António Coimbra Rodrigues da Silva, Professor Associado com Agregação do Departamento de Química da Universidade de Aveiro e do Doutor Jorge Manuel Alexandre Saraiva, Investigador Auxiliar do Departamento de Química da Universidade de Aveiro.

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Aos meus Pais

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Palavras-chave

'Rainha Cláudia Verde', *Prunus domestica* L., Denominação de Origem Protegida, confitagem, polissacarídeos, textura, microestrutura, compostos voláteis, compostos fenólicos, enzimas, parede celular

Resumo

As Ameixas d'Elvas são ameixas (*Prunus domestica* L.) da variedade 'Rainha Cláudia Verde' produzidas numa área geográfica delimitada do Alto Alentejo e detentoras de uma Denominação de Origem Protegida (DOP) reconhecida pela União Europeia. A confitagem deste fruto, por um processo tradicional, origina um produto de grande valor acrescentado, denominado de Ameixa d'Elvas confitada. O processo da confitagem da Ameixa d'Elvas consiste num cozimento dos frutos inteiros, colhidos num grau de maturação bem definido (16-17 ºBrix e 1 meq de ácido málico por 100 g de fruto fresco) durante 15 minutos e em seguida as ameixas são colocadas numa calda de açúcar que vai sendo concentrada sucessivamente até atingir os 75 ºBrix. As ameixas são mantidas nesta calda durante, pelo menos, 2 meses.

O principal objectivo deste trabalho foi a compreensão e resolução de um problema industrial na confitagem da Ameixa d'Elvas. O problema consiste na impossibilidade de confitar ameixas de um pomar (Cano) localizado na região delimitada pela DOP, pois os frutos perdem a integridade com o cozimento. Com este intuito, as Ameixas d'Elvas foram caracterizadas a nível físico-químico e as transformações bioquímicas, químicas e físicas que ocorrem com a confitagem foram estudadas em ameixas provenientes de dois pomares, Vila Viçosa e Cano. Os frutos provenientes do pomar de Vila Viçosa dão origem a um produto final de boa qualidade, que contrasta com os frutos do Cano.

Os principais compostos fenólicos presentes na polpa da ameixa em fresco são os flavan-3-óis (procianidinas) e os ácidos hidroxicinâmicos. A pele da ameixa contém ainda outra classe de compostos, os flavonóis. As Ameixas d'Elvas analisadas são constituídas por uma maior quantidade de compostos fenólicos, nomeadamente procianidinas com maior grau de polimerização, em comparação com as ameixas de outras variedades e espécie e também com ameixas da mesma variedade produzidas fora da região delimitada pelo DOP.

O aroma característico da Ameixa d'Elvas confitada tem a sua origem nos compostos voláteis provenientes da ameixa em fresco e formados durante o processo de confitagem, nomeadamente o octanoato de etilo, o nonanal, o eugenol, o acetato de 2-feniletilo, o linalol, o benzoato de etilo, o benzaldeído, o 2-heptenal, o ácido hexadecanóico, o ácido 3-metilbutanóico e o β-citronelol. A presença de todos estes compostos, à excepção do 2-heptenal, na calda de açúcar mostra que esta tem também um importante contributo para o aroma da Ameixa d'Elvas confitada.

A análise da estrutura e organização celular da polpa das ameixas não processadas revelou que os tecidos do parênquima são constituídos por células isodiamétricas com uma área entre 4 e 6 μm^2 e espaços intercelulares reduzidos (0,02-0,05 μm^2), conferindo rigidez ao fruto.

O tratamento térmico provoca uma degradação da lamela média e a perda da organização celular, originando uma diminuição na firmeza (80-94%), rigidez (93-98%) e trabalho necessário para a deformação dos tecidos (72-91%). A imersão das ameixas na calda de açúcar promove um aumento nos parâmetros de textura analisados para valores próximos aos da polpa da ameixa não processada. Esta recuperação da consistência do fruto está relacionada com um aumento da adesão intercelular e a recuperação da estrutura das células do parênquima.

As paredes celulares da polpa da ameixa são constituídas principalmente por polissacarídeos pécticos (62%) e celulose (15%). Estes polímeros são degradados e solubilizados com o tratamento térmico, o que explica a perda de adesão celular e da textura dos frutos. Por sua vez, o aumento da adesão intercelular com a confitagem está relacionada com os polissacarídeos pécticos solúveis da parede celular. Estes polissacarídeos possuem um elevado grau de esterificação (83%). Na presença da sacarose, que difunde para o interior do fruto por osmose durante a confitagem, a sua gelificação contribui para o aumento da adesão celular e consistência do fruto.

No decurso do trabalho, foi proposta uma metodologia simples, fiável e limpa para a determinação simultânea do grau de esterificação e acetilação dos polissacarídeos da parede celular. Após saponificação das amostras de polissacarídeos, o metanol e o ácido acético libertado para a fase de vapor é analisado por microextracção em fase sólida e cromatografia em fase gasosa.

O presente estudo permitiu observar que em ameixas com um grau de maturação mais avançado ocorre uma maior degradação da microestrutura dos tecidos e uma maior diminuição nos parâmetros de textura com o tratamento térmico e uma menor recuperação com a confitagem. Este facto está relacionado com a maior degradação dos polissacarídeos da parede celular devido à acção das enzimas pectina metilesterase (PME), poligalacturonase e celulase durante o amadurecimento. A PME foi ainda purificada e caracterizada a nível bioquímico, mostrando ser relativamente estável a tratamentos térmicos (5 minutos a 50-60 ºC) e de alta pressão (≤ 600 MPa), pelo que a sua actividade terá que ser tida em consideração durante o processamento da ameixa.

As ameixas não processadas do pomar do Cano revelaram uma maior solubilização dos polissacarídeos da parede celular em comparação com as de Vila Viçosa, influenciando o seu comportamento durante o processamento. Esta solubilização dos polissacarídeos está relacionada com uma maior actividade das enzimas poligalacturonase (44%) e celulase (55%) quando comparada com a actividade destas enzimas nas ameixas colhidas em Vila Viçosa para o mesmo estado de maturação definido de acordo com os sólidos solúveis totais e acidez titulável. Estes resultados são indicativos de que as ameixas do pomar do Cano estão, no que se refere à estrutura e composição dos polissacarídeos das paredes celulares, num estado de maturação mais adiantado do que as de Vila Viçosa. Este estudo mostra que os parâmetros convencionais usados para determinar a maturação das ameixas não são adequados para seleccionar as ameixas para confitar. Decorre desta tese de doutoramento que o conteúdo em ácido urónico no resíduo insolúvel em álcool da polpa das ameixas seja a metodologia a usar para avaliar o estado de maturação das ameixas para confitar em todos os pomares da região DOP.

Keywords

'Rainha Cláudia Verde', *Prunus domestica* L., Protected Designation of Origin, candying, polysaccharides, texture, microstructure, volatile compounds, phenolic compounds, enzymes, cell wall

Abstract

Plums (*Prunus domestica* L.) of 'Rainha Cláudia Verde' variety, produced in a defined region of the South-East of Portugal, have a Protected Designation of Origin (PDO), "Ameixa d'Elvas", recognized by European Union. A high added value product can be obtained by a traditional candying, named by "Ameixa d'Elvas" candied plum. The candying process consists in boiling the intact plums, collected in a defined maturation point (16 -17 ºBrix and 1.0 meq malic acid/100 g fruit flesh weight), in water for 15 min and further immersion in a sucrose syrup, which is successively concentrated until 75 ºBrix. The plums are kept for two months in the sucrose syrup.

The main aim of the present work was to understand and solve one industrial problem during the candying process of "Ameixa d'Elvas" plums. Plums from a specific orchard in the PDO region (Cano) can not be processed, since the fruits showed an appreciable loss of tissue consistency and skin disruption after boiling. This purpose led to the study of some physical and chemical characteristics of plums and the changes that occurred along candying process. The plums studied were collected in two orchards, Vila Viçosa and Cano. Plums from Vila Viçosa orchard originate a product with good quality, contrarily to the plums from Cano orchard.

The main phenolic compounds present in the plum flesh are flavan-3-ols (procyanidins) and hydroxycinnamic acids. In the skin, another class of compounds are present, the flavonols. A higher amount of phenolic compounds were present in the "Ameixa d'Elvas" plums analysed, mainly procyanidins with a higher degree of polymerization, when compared to plums of other varieties and specie and also to plums of the same variety produced outside the PDO delimited region.

The specific aroma of "Ameixa d'Elvas" plums arise from the fresh fruit volatile compounds and from the compounds produced during the heat processing, namely ethyl octanoate, nonanal, eugenol, 2-phenylethylacetate, linalool, ethyl benzoate, benzaldehyde, 2-heptenal, hexadecanoic acid, 3-methyl butanoic acid, and β-citronellol. The presence of these compounds, except 2-heptenal, in the sucrose syrup shows the contribution of the syrup for the overall aroma of the candied plum.

Microstructure analysis of the unprocessed plums revealed that the parenchyma tissue is constituted by isodiametric (4 and 6 μm^2 of area) and thickly packed cells (0.02-0.05 μm^2 of intercellular area), which originate a moderately hard texture. The tissue softening, visible by the decrease of firmness (80-94%), rigidity (93-98%), and deformation work (72-91%), occurring with thermal treatment was due to the middle lamella degradation. After candying a recovery in the texture parameters is observed to values similar of fresh tissue. The recovery in tissue consistency is related to the increase of parenchyma intercellular adhesion and the recuperation of the parenchyma cells structure.

Plums cell walls are composed mainly by pectic polysaccharides (62%) and cellulose (15%). During the boiling step, these polysaccharides are degraded and solubilised, which are related to the loss of intercellular adhesion and tissue softening. The presence of soluble and highly esterified (83%) pectic polysaccharides allows their gelification inside the fruits in the presence of sucrose, which diffuses by osmosis during candying process, leading to the recovery of fruits consistency and intercellular adhesion.

Through the work, a simple, reliable and clean methodology was proposed for the simultaneous determination of the esterification and acetylation degree of cell wall polysaccharides. After saponification of the polysaccharides samples, the methanol and the acetic acid released to the vapour phase are simultaneously extracted by solid phase microextraction and analysed by gas chromatography.

This study allowed to observe that plums in an advanced ripening stage have a more pronounced degradation with boiling and lower recovery of cells shape, size and texture characteristics. The higher activity of the enzymes, pectin methylesterase (PME), polygalacturonase, and cellulase of fresh fruits along ripening are related to the observed higher extension of degradation of cell wall polysaccharides after boiling. PME was also purified and biochemically characterised, showing to be relatively stable to thermal (5 min at 50-60 ºC) and high pressure (≤ 600 MPa) treatments, which infer that its activity should be take in account during the plum processing.

Unprocessed plums from Cano orchard show a higher solubilisation and depolimerisation of cell wall polysaccharides than Vila Viçosa plums, which influence their behaviour along processing. The higher solubilisation of the polysaccharides is related to the higher activity of the polygalacturonase (44%) and cellulase (55%), comparing to the enzyme activity of plums harvested in Vila Viçosa with the same stage of ripening evaluated by total soluble solids and titratable acidity. These results permitted to infer that plums from Cano orchard are in a more advanced stage of ripening, in relation to the structure and composition of cell wall polysaccharides, comparing to Vila Viçosa ones. This study shows that conventional parameters used to evaluate the stage of ripening of plums are not adequate to select the fruits for candying. The results presented in this thesis allowed to conclude that uronic acids content in the alcohol insoluble residue of the flesh plums should be the methodology used to evaluate the stage of ripening of the plums for processing, from all orchards within PDO region.

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CAPÍTULO I

INTRODUÇÃO GERAL E ENQUADRAMENTO DO TRABALHO

1. AMEIXA

A ameixa é um dos frutos mais consumidos e comercializados a nível mundial (FAO, 2006). Este fruto é consumido em fresco e também é usado para a produção de ameixas secas, em calda e de bebidas. A ameixa é uma drupa, que pertence à mesma família botânica (Rosáceas) do pêssego, do damasco e da cereja. Estes frutos são muito apreciados pelos consumidores devido principalmente ao seu sabor doce e às suas características agradáveis de aroma e cor.

A produção mundial de ameixas foi estimada, em 2005, em aproximadamente 8,9 milhões de toneladas (FAO, 2006). Os principais países produtores de ameixa são a China, a Roménia, os Estados Unidos da América, o Chile, a Turquia e a França. Mais de 50% da produção mundial concentra-se no continente asiático, cabendo a liderança à China com 4,6 milhões de toneladas. A Europa tem uma quota na produção mundial de 24% e a produção Portuguesa representa cerca de 1% da produção de ameixas da Europa (Tabela 1).

Em Portugal, a ameixa tem pouco peso na produção total de frutos frescos, não chegando aos 2%. Contudo, é um fruto com algum sucesso a nível internacional, em que o volume de vendas ao exterior representa cerca de 13% da produção nacional de ameixa (média dos anos 2000 a 2004). Os pomares de ameixeiras ocupam uma área total de 1 904 hectares em Portugal continental, sendo em geral muito reduzida a sua dimensão média (0,3 hectares), com uma produção associada de 16 149 toneladas de ameixas em 2005 (INE, 2005).

O Ribatejo e Oeste é a principal região Portuguesa de produção de ameixa, com cerca de 50% da área e da produção do Continente, seguida do Alentejo com 25% da área e 28% da produção de ameixa (média dos anos 2000 a 2004). Numa década a região do Alentejo aumentou 10% a sua área de produção e conseguiu uma melhoria acentuada no rendimento da cultura, em comparação com as outras regiões (INE, 2005).

Tabela 1 – Produção mundial de ameixas em 2005 (FAO, 2006).

Países	*Produção em 2005 (1000 toneladas)*
África	**239**
África do Sul	54
Argélia	46
Marrocos	61
América	**923**
Argentina	132
Chile	255
Estados Unidos da América	412
México	76
Ásia	**5603**
China	4629
Índia	98
República da Coreia	76
República Islâmica do Irão	146
Turquia	220
Uzbequistão	121
Europa	**2132**
Áustria	62
Bósnia e Herzegovina	96
Espanha	191
França	214
Itália	185
Polónia	91
Portugal	16
Roménia	622
Rússia	185
Ucrânia	166
Oceânia	**35**
Austrália	33
Nova Zelândia	2
Total mundial	***8932***

1.1. Descrição botânica e características

A ameixa pertence à subfamília das Prunoídeas e ao género *Prunus*. As ameixas economicamente mais importantes podem ser divididas em três grupos de acordo com o local onde são produzidas: as europeias, as japonesas e as americanas.

As ameixas europeias são das espécies *Prunus domestica* e *Prunus insititia*, sendo a primeira espécie a mais importante a nível comercial. As ameixas da espécie *Prunus domestica* podem ter cor, tamanho e forma muito variada consoante a variedade dos frutos. Devido a esta diversidade, as ameixas desta espécie foram subdivididas em quatro grupos, de acordo com a sua cor, formando os grupos das ameixas verdes, amarelas e vermelhas, e também do ponto de vista comercial, originando o grupo das ameixas secas. Este último grupo é constituído por ameixas com um teor em açúcares elevado e que são caracterizadas por serem ovais com cor púrpura e uma polpa com textura firme. As ameixas denominadas de verdes, onde se enquadra a variedade 'Rainha Cláudia Verde', são principalmente consumidas em fresco ou em calda. Os frutos são descritos como redondos com uma polpa doce e sumarenta e, apesar de serem denominados de verdes, a sua cor pode variar entre o verde e o amarelo ou até ligeiramente vermelho. Os grupos das ameixas amarelas e vermelhas são pouco importantes a nível económico, sendo normalmente usadas apenas para produzir produtos processados, como sumos e compotas. As ameixas europeias da espécie *Prunus insititia* são caracterizadas por serem pequenas, menos de 3 cm de diâmetro, redondas e de cor púrpura ou amarela. Usualmente, estas ameixas são usadas para a produção de compotas (Bhutani e Joshi, 1995).

As ameixas japonesas são da espécie *Prunus salicina* e podem ter diversos tamanhos e formas. A pele dos frutos é fina e adstringente, podendo ter cor amarela, verde ou vermelha. A polpa é caracterizada por ser firme, fibrosa e de cor amarela, vermelha ou roxa.

A maioria das ameixas denominadas de americanas são de variedades híbridas. Estes frutos têm a pele de cor amarela ou laranja e a polpa amarela dourada. Este grupo de ameixas tem pouca importância a nível comercial. Os frutos são normalmente usados para processar, nomeadamente para a obtenção de compotas, sendo pouco consumidos em fresco (Bhutani e Joshi, 1995).

1.2. Anatomia do fruto

A nível morfológico, as ameixas são drupas caracterizadas por um endocarpo ou caroço muito lenhificado que contém no interior a semente. A função do caroço é a de

conservar a semente que existe no seu interior, que por sua vez é constituída por células cuja função é armazenar energia e nutrientes suficientes para uma posterior germinação. A ameixa é constituída por um mesocarpo (ou polpa) volumoso e suculento. Na polpa são também visíveis os feixes vasculares (Figura 1). O fruto é envolvido por uma epiderme ou pele muito fina, que na parte exterior é revestida por uma camada de cutina, um polímero cuja função é reduzir a perda de água dos tecidos.

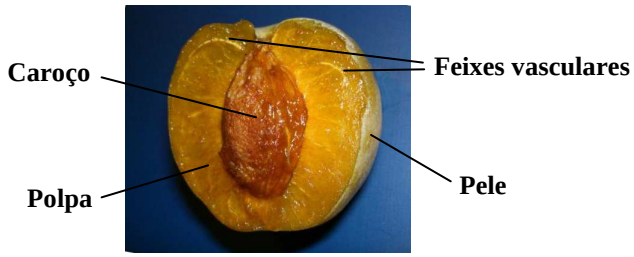

Figura 1 – Morfologia da ameixa.

A nível microscópico não existem dados relativos à composição dos tecidos da polpa da ameixa, sendo provável que seja semelhante à de outros frutos da mesma família botânica. A polpa dos frutos é maioritariamente constituída por células do parênquima com paredes celulares finas e por feixes vasculares. Estes feixes são constituídos pelas células de xilema e floema, que têm como principal função a condução da água e dos nutrientes, respectivamente, por todo o fruto. As células dos feixes vasculares têm as paredes mais espessas, que se tornam mais lenhificadas com o amadurecimento do fruto, contribuindo também para a resistência dos tecidos.

1.3. Maturação da ameixa

A ameixa é um fruto climatérico e, portanto, durante a fase final de amadurecimento, apresenta um pico de produção de etileno, acompanhado pelo aumento súbito da actividade respiratória. O etileno é um composto volátil, sintetizado naturalmente pelo fruto à medida que este amadurece. É considerado uma hormona que estimula o processo de maturação do fruto. Os frutos climatéricos, como a ameixa, podem amadurecer após terem sido colhidos,

apresentando praticamente as mesmas características que os frutos amadurecidos na árvore (Eskin, 1990).

O avanço da maturação origina um aumento do teor em sólidos solúveis totais devido principalmente aos açúcares. A sacarose aparece apenas na última fase de maturação da ameixa e a sua concentração aumenta para valores superiores aos açúcares redutores (Bhutani e Joshi, 1995). Simultaneamente, ocorre a diminuição da acidez devido à diminuição da quantidade de ácidos orgânicos. É neste período que ocorre também o amolecimento do fruto em conjunto com as mudanças de coloração. Assim, os parâmetros de maturação utilizados para avaliar a altura correcta para a colheita das ameixas estão fundamentados em métodos físicos, químicos, fisiológicos ou combinações entre eles, os quais permitem monitorizar o avanço da maturação. Os mais utilizados na ameixa são: o conteúdo em sólidos solúveis, a acidez titulável, a razão entre os sólidos solúveis e os ácidos, a firmeza da polpa e a cor do fruto (Taylor *et al.*, 1995; Abdi *et al.*, 1997).

Cada um destes parâmetros, de forma isolada, pode ser afectado pelas condições de cultivo do pomar. Para diminuir essa variabilidade, nos testes de maturação, devem ser sempre considerados dois ou três parâmetros de forma conjunta (Taylor *et al.*, 1995; Abdi *et al.*, 1997). Os parâmetros usados para determinar o estado de maturação óptimo dependem também da variedade das ameixas e do fim a que se destinam. A determinação do ponto óptimo de colheita é de extrema importância pois permite assegurar uma boa qualidade do fruto, quer seja consumido em fresco quer seja processado.

1.4. Processamento da ameixa

O principal objectivo do processamento de frutos é a sua preservação numa forma estável, para serem consumidos durante um longo período de tempo e serem também facilmente transportados para longas distâncias. O processamento altera as características do fruto e permite produzir diversos produtos para consumo directo ou para utilizar na preparação de outro tipo de alimentos.

Usualmente, as ameixas com alto teor em açúcar e com polpa firme são usadas para serem processadas. Uma grande percentagem das ameixas processadas são desidratadas

para obter ameixa seca. Algumas são colocadas em calda de açúcar e enlatadas e uma pequena percentagem é usada para a preparação de compota, sumo e bebidas fermentadas de ameixa.

1.4.1. Tipos de processamento

Ameixa seca

As ameixas secas são produzidas principalmente nos Estados Unidos da América (Califórnia), que detém cerca de 70 a 75% do consumo mundial. Antigamente, o método de processamento mais usual era a secagem ao sol. O objectivo principal era evitar o aparecimento de fungos ou bolores nos frutos durante o período das chuvas por redução da actividade de água. Hoje em dia, as ameixas previamente lavadas com água são colocadas em túneis de ar forçado (70 ºC) para a sua desidratação até uma percentagem de humidade final de 17 a 19%. As ameixas podem também ser descaroçadas antes da secagem para a obtenção de ameixa seca sem caroço. Algumas ameixas secas podem ser desidratadas em vácuo até valores muito reduzidos de humidade (4%). As ameixas secas podem também ser usadas para produzir outros produtos como sumos de ameixa seca, concentrados de sumo e ameixas secas em lata (Somogyl, 1996).

A desidratação osmótica é um processo de secagem de frutos em que pedaços ou fatias de ameixa são colocadas em soluções concentradas de açúcar (sacarose ou glucose) para remover cerca de 50% de humidade do fruto por osmose. Para aumentar a velocidade de desidratação, o processo de osmose pode ocorrer sob vácuo. Os frutos parcialmente desidratados podem depois ser completamente secos por um processo convencional de secagem. Nos frutos inteiros, um tratamento térmico prévio degrada a cutícula exterior da pele da ameixa, aumentando a velocidade de desidratação e diminuindo o tempo de secagem. A desidratação osmótica tem diversas vantagens em relação à secagem convencional: diminui o tempo em que o fruto está exposto a temperaturas elevadas, reduz a degradação da cor, do sabor e da acidez de alguns frutos e permite a incorporação de algum açúcar. As ameixas secas por desidratação osmótica têm características físico-químicas e propriedades sensoriais que aumentam a aceitabilidade do produto final pelo consumidor (Bhutani e Joshi, 1995).

Ameixa em lata

A ameixa conservada em lata é também um produto industrialmente importante no processamento das ameixas, embora seja muito menos importante do que os pêssegos ou as pêras. O principal objectivo deste tipo de processamento é a destruição dos microorganismos pelo tratamento térmico (esterilização), permitindo a conservação do fruto por longos períodos de tempo.

O processamento consiste em seleccionar e lavar os frutos que foram colhidos numa fase precoce de maturação em que a cor ainda não está completamente desenvolvida e o fruto tem uma consistência firme. Os frutos depois de descascados são colocados em latas ou frascos de vidro e uma solução concentrada de sacarose é adicionada a quente, entre 88 e 93 ºC. A solução de açúcar tem usualmente 20% de sacarose, mas pode atingir os 55%. As latas ou frascos depois de fechados são aquecidos a 100 ºC durante 10 a 25 minutos, sendo depois arrefecidos a 38 ºC. As ameixas, durante o processamento e armazenamento, vão absorver algum açúcar da solução onde estão mergulhadas (Bhutani e Joshi, 1995).

As ameixas secas podem também ser conservadas em lata. O processamento é idêntico ao das ameixas em fresco mas a única diferença é que as ameixas secas são fervidas em água durante 4 a 5 minutos antes de serem colocadas na lata e a solução de sacarose tem no máximo 30 ºBrix (Somogyl, 1996).

Nos testes sensoriais do produto final, ameixas mais firmes e com uma cor mais intensa são preferidas. O aumento da textura dos frutos pode ser conseguido adicionando cloreto de cálcio à solução onde as ameixas são processadas. O aumento da firmeza dos frutos está relacionado com a complexação dos iões bivalentes de cálcio com os grupos carboxílicos dos polissacarídeos pécticos. A cor dos frutos depois do processamento está relacionada com o elevado conteúdo em antocianinas das ameixas em fresco. As antocianinas das ameixas difundem para a solução durante o processamento, pelo que a estabilidade da cor melhora se a quantidade de líquido que envolve os frutos for menor. Por esta razão as ameixas em lata são produzidas com muito pouca quantidade de solução de sacarose para a obtenção de um produto final com melhores características sensoriais (Bhutani e Joshi, 1995).

Compota

Outro método de conservação da ameixa é a sua transformação em compota, isoladamente ou juntamente com outros frutos. O procedimento é semelhante ao de outros frutos. O fruto, depois de descascado e descaroçado, é colocado em pouca água juntamente com a sacarose. Os frutos normalmente usados para compota são de variedades que amadurecem mais tarde, pois têm um balanço de ácidos e açúcar mais apropriado e um maior conteúdo em matéria seca (Bhutani e Joshi, 1995), para além de polissacarídeos pécticos mais esterificados.

Sumo

O sumo de ameixa é pouco vulgar, sendo mais usado para adicionar a sumos de outros frutos. O método de preparação do sumo é o convencional para a preparação de sumos de fruta. As ameixas são prensadas e em seguida o sumo é filtrado. Normalmente o sumo é pasteurizado por um tratamento térmico a temperaturas entre os 80 e 90ºC antes e/ou depois de ser embalado. Podem também ser adicionadas enzimas pécticas, que actuam durante 6 a 12 horas antes da filtração do sumo. Estas enzimas hidrolisam os polissacarídeos pécticos, provocando um aumento do rendimento e facilitando a filtração do sumo. O uso das enzimas origina um sumo com menor viscosidade, menos turvo e com melhor cor, sem alterar o sabor. Para além do método de extracção usado, o estado de maturação e as condições de produção da ameixa vão também influenciar a composição do sumo (Bhutani e Joshi, 1995).

Os concentrados de sumo de ameixa podem ser preparados por evaporação do sumo até atingir os 60 ºBrix, à pressão atmosférica, ou aquecendo a 50-60 ºC sob vácuo até 73 ºBrix. Esta concentração aumenta os sólidos solúveis totais, os açúcares redutores, o escurecimento e a viscosidade do sumo, mas diminui a sua acidez (Bhutani e Joshi, 1995).

Nos Estados Unidos é muito usado o sumo de ameixa seca, que não é um sumo convencional de fruta pois é obtido por extracção com água. O processamento consiste em ferver as ameixas secas desfeitas em água, durante 3 a 8 horas, ou fazer extracções sucessivas com água quente, em que cada extracção tem uma duração de 2 a 4 horas. Em ambos os processos ocorre a difusão dos compostos para a água, que é posteriormente filtrada e concentrada até 19 a 21 ºBrix para a obtenção do sumo. Os concentrados de sumo

de ameixa seca são preparados a partir do sumo obtido por adição de enzimas pécticas para a degradação das pectinas e diminuição da viscosidade. O sumo depois de filtrado é concentrado sob vácuo até 60 ºBrix a temperaturas inferiores a 48 ºC (Somogyl, 1996).

Bebidas fermentadas de ameixa

As ameixas podem dar origem a uma bebida alcoólica por fermentação da polpa. Esta bebida de ameixa é popular em alguns países como a Alemanha. A preparação consiste em adicionar água a ameixas completamente maduras numa proporção de 1:1, em que a fermentação alcoólica ocorre por adição de leveduras, principalmente *Saccharomyces cerevisiae* e *Schizosaccharomyces pombe*. Podem também ser adicionadas enzimas pécticas para aumentar a quantidade de sumo obtido e ajudar na posterior clarificação da bebida. Esta mistura é deixada a fermentar durante 8 a 10 dias, sendo depois prensada. Uma vez que as ameixas têm baixo teor em açúcares em comparação com as uvas, pode ser adicionado açúcar ao sumo para aumentar o teor alcoólico, dependendo do tipo de bebida que se pretende. O restante processamento é idêntico ao do vinho convencional. As variedades de ameixa ‘Santa Rosa’ e ‘Rainha Cláudia Verde’ são as que apresentam melhores qualidades do ponto de vista sensorial.

2. AMEIXA D'ELVAS

A Ameixa d'Elvas é um produto regional detentor de Denominação de Origem Protegida (DOP) segundo o Despacho nº 49/94, de 20 de Janeiro, e publicado no Diário da República nº 28 de 3 de Fevereiro de 1994 (II Série). Reconhecida pela União Europeia, a Denominação de Origem está registada e protegida pelo Regulamento nº 1107/96, de 12 de Junho de 1996, relativo à protecção das indicações geográficas e das denominações de origem dos produtos agrícolas e dos géneros alimentícios (DGDR, 2001).

O estatuto de Organismo Privado de Controlo e Certificação foi reconhecido à APAAA — Associação de Produtores de Ameixa do Alto Alentejo. A organização de produtores Fruteco - Fruticultura Integrada Lda., com sede em Estremoz, é a entidade gestora desta DOP, cujo objectivo principal é a concentração, tratamento da produção local da variedade de ameixa tradicional na região e a sua comercialização.

2.1. Características

As Ameixas d'Elvas são frutos da espécie *Prunus domestica* L. e da variedade 'Rainha Cláudia Verde', produzidos, transformados e acondicionados no Alto Alentejo, numa área geográfica delimitada dos distritos de Portalegre e Évora (Figura 2), que inclui todas as freguesias dos concelhos de Borba, Campo Maior e Vila Viçosa e algumas freguesias dos concelhos de Elvas, Estremoz, Sousel, Monforte e Alandroal (Anexo II, D.R. nº 28 de 3/2/1994).

As Ameixas d'Elvas podem ser comercializadas em fresco ou transformadas por processos tradicionais de secagem ou confitagem. As características das ameixas variam conforme o modo como se apresentam (Anexo I, D.R. nº 28 de 3/2/1994).

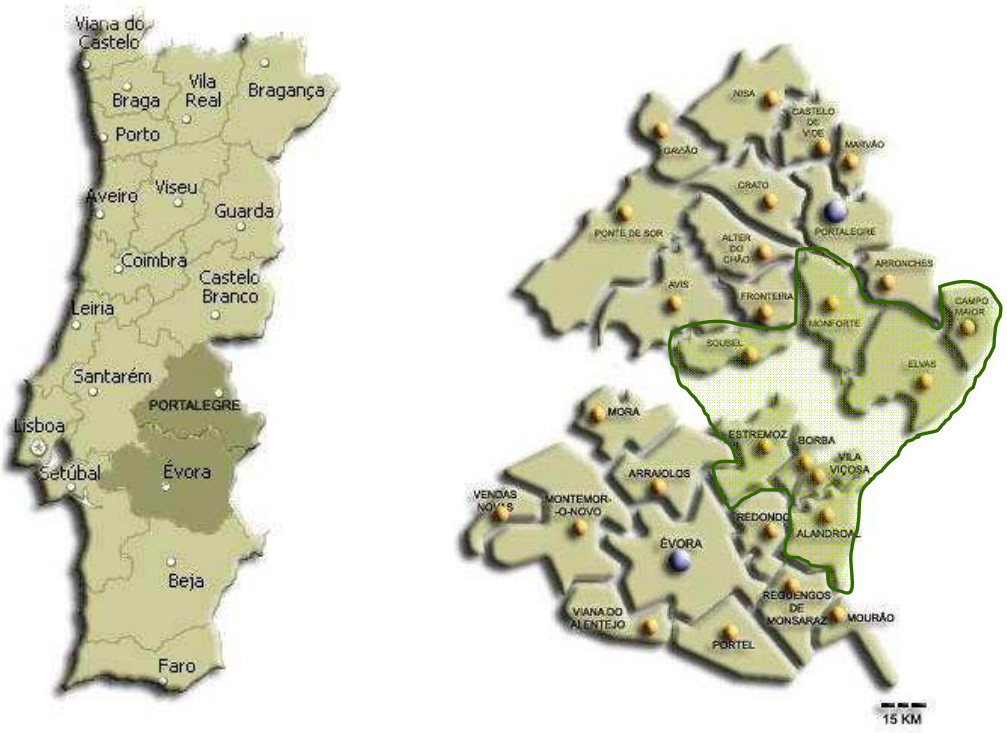

Figura 2 – Mapa de Portugal e dos distritos de Portalegre e Évora.

A Ameixa d'Elvas fresca é descrita como um fruto de forma arredondada, ligeiramente achatada nos pólos, com um diâmetro equatorial mínimo de 32 mm. A ameixa, completamente revestida de pruína, tem uma cor verde podendo no entanto apresentar leves tons amarelados e rosados (Figura 3). O fruto maduro tem que ter um teor de açúcar de, pelo menos, 18 ºBrix. A polpa tem que se apresentar muito suculenta, com um aroma intenso característico e uma consistência de, pelo menos, 6 kg/cm^2. O caroço é pequeno, não pode ultrapassar 1,1 g, e tem que se destacar facilmente da polpa (Anexo I, D.R. nº 28 de 3/2/1994).

Figura 3 – Ameixa d'Elvas fresca.

A Ameixa d'Elvas pode ser desidratada por exposição ao sol, obtendo-se um produto que é denominado por passa de Ameixa d'Elvas. Esta é produzida a partir do fruto inteiro isento de ataques de parasitas, de doenças e de matérias estranhas, e completamente maduro, e tem que possuir em média 23 ºBrix. Depois da desidratação do fruto, este deve apresentar um teor de humidade inferior a 23% e um teor de açúcares totais superior a 67%. O fruto tem que ter uma cor castanho-escura característica e com brilho, sem fendas na epiderme que ponham a descoberto mais de 5 mm de polpa e sem vestígios do pedúnculo (Figura 4). Estes frutos têm que apresentar ainda um espessamento da polpa e da pele uniforme, inferior a 10 mm, com uma consistência "de média a dura" e com sabor doce "característico" (Anexo I, D.R. nº 28 de 3/2/1994).

Figura 4 – Passa de Ameixa d'Elvas.

A Ameixa d'Elvas confitada consiste no fruto inteiro obtido pelo processo tradicional de confitagem, a partir de frutos frescos com 18 ºBrix e uma consistência de 5 kg/cm^2. Os frutos confitados têm que ter cor esverdeada com tonalidades castanhas e um cheiro e sabor característicos da variedade 'Rainha Cláudia Verde'. O teor mínimo de açúcares totais dos frutos confitados é de 70%. Os frutos confitados podem ser comercializadas sob três formas: escorrida (Figura 5), em calda e com cobertura de açúcar. Para acondicionar as ameixas confitadas podem ser usadas caixas de cartão, madeira ou cortiça, com formas redondas e rectangulares e para a ameixa em calda usam-se frascos de vidro (Anexo I, D.R. nº 28 de 3/2/1994).

Figura 5 – Ameixa d'Elvas confitada.

2.2. Cultivo da ameixa

A variedade de ameixeira 'Rainha Cláudia Verde', cultivada no Alto Alentejo, adquiriu uma especificidade própria devido às condições do solo e clima da região. Estas condições permitem a produção de uma ameixa com características únicas, diferente da produzida em outras regiões de Portugal. Em geral, a ameixeira é uma planta de clima temperado que entra em dormência no Inverno devido às baixas temperaturas. Necessita, para o seu desenvolvimento vegetativo e produtivo, de Invernos frios, Primaveras amenas e bem ensolaradas e Verões quentes e secos, a fim de favorecer a sanidade e coloração dos frutos. O frio é classificado como o parâmetro de maior importância na produção de ameixa com qualidade, tanto para eliminar a dormência, como após a floração. Quando as necessidades de frio não são satisfeitas, ocorre uma floração pouco uniforme e insuficiente. As temperaturas elevadas durante a maturação possibilitam a obtenção de frutos muito ricos em açúcares (Barroso, 1990).

A maioria dos pomares produtores de Ameixa d'Elvas são controlados a nível agronómico com clones de 'Rainha Cláudia Verde' que foram previamente seleccionados para a região. O estudo da biologia floral e exigências de polinização da variedade e, mais recentemente, a introdução de novos porta-enxertos, permitiu encontrar as condições que melhor se adaptavam à região do Alto Alentejo (Barroso, 1990). A qualidade do fruto decide-se precocemente no pomar, ou seja, por mais eficientes e cuidadas que sejam a colheita e armazenagem não se consegue um fruto de boa qualidade se os factores que influenciam a cultura da ameixa (rega, fertilização, porta-enxertos, etc.), não forem bem controlados. A produtividade desta ameixa é baixa, ronda as 6 a 7 toneladas por hectare,

em comparação com a média nacional (8,4 ton/ha), mas em compensação a sua qualidade permite a sua valorização na comercialização.

A colheita da ameixa é realizada durante o mês de Julho e a primeira semana de Agosto. Os frutos para consumo em fresco são colhidos na pré-maturação, com uma média de 18 ºBrix. O período ideal de colheita corresponde à altura em que o fruto, inicialmente verde, começa a adquirir tons rosados e amarelados característicos. Nestas condições, podem conservar-se durante três semanas no frigorífico e mais de uma semana à temperatura ambiente, evoluindo naturalmente sem perda aparente de qualidade.

2.3. Processamento da Ameixa d'Elvas confitada

Os frutos usados para confitar são colhidos um pouco antes dos frutos para consumo em fresco, possuindo um máximo de 18 ºBrix. Cerca de 20% da produção de ameixas 'Rainha Cláudia Verde', no Alto Alentejo, é usada na indústria para a obtenção de ameixa confitada (INE, 2005).

As Ameixas d'Elvas confitadas são obtidas pelo processo tradicional, o que garante características únicas a este produto. A Confibor – Transformação Agro-alimentar Lda., com sede em Estremoz, é a principal empresa que produz a Ameixa d'Elvas confitada. Nesta empresa as ameixas da variedade 'Rainha Cláudia Verde' são processadas segundo a receita tradicional, devidamente adaptada ao processo industrial e às exigências higieno-sanitárias.

O processamento da ameixa consiste em cozer os frutos, que foram previamente seleccionados e lavados, em água a 90 ºC até flutuarem à superfície da água, o que demora aproximadamente 15 minutos (Figura 6a). Depois de arrefecerem, as ameixas cozidas, são colocadas numa calda de açúcar com 60 ºBrix. No dia seguinte, a calda é concentrada até 65 ºBrix e ao fim de uma semana é novamente concentrada até 75 ºBrix. As ameixas são mantidas nesta calda a 75 ºBrix durante, pelo menos, 2 meses (Figura 6b). A calda de açúcar é, quando necessário, concentrada para manter os 75 ºBrix. Para a concentração da calda, esta é retirada dos tanques, aquecida a 60-70 ºC, podendo ser-lhe também

adicionada sacarose, e depois de convenientemente concentrada a calda é adicionada às ameixas ainda quente.

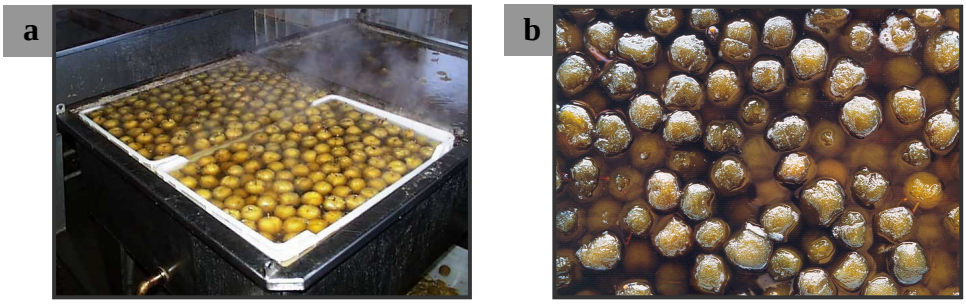

Figura 6 – Processamento de confitagem da Ameixa d'Elvas: a) cozimento, b) ameixas na calda de açúcar.

Ao fim de 2 meses, as ameixas confitadas são retiradas da calda, lavadas e escorridas para serem embaladas em caixas de cartão ou de cortiça (embalagem tradicional). O processo de embalagem é feito manualmente pois a ameixa tem de ficar sempre com o pedúnculo virado para cima. Podem também ser comercializadas em frascos de vidro com a calda de açúcar (Figura 7).

Figura 7 – Ameixas d'Elvas confitadas embaladas pela Confibor.

3. ENQUADRAMENTO DO TRABALHO

A Ameixa d'Elvas é um dos produtos específicos regionais detentor de uma Denominação de Origem Protegida (DOP) que se encontra em fase de consolidação nos sistemas agrícolas do Alto Alentejo e começa a contribuir para uma actividade económica sustentável nesta região. A transformação deste fruto por confitagem origina um produto de grande valor acrescentado e de qualidade reconhecida a nível nacional e internacional - a Ameixa d'Elvas confitada.

Este trabalho de Doutoramento visou o estudo das alterações que ocorrem na Ameixa d'Elvas durante a confitagem. A ideia para a realização do trabalho surgiu da ocorrência sistemática de um problema a nível industrial na confitagem da Ameixa d'Elvas e da necessidade de optimizar a produção industrial da ameixa confitada. O problema reside na impossibilidade de processar ameixas de um pomar específico, o pomar do Cano, dado que os frutos perdem a integridade durante o processamento térmico aplicado no processo de confitagem. Apesar das ameixas deste pomar terem aparentemente as mesmas características das outras Ameixas d'Elvas e o pomar se situar na região definida para a produção deste produto DOP, estes frutos originam importantes perdas económicas, pois os frutos confitados ficam sem valor comercial.

Neste contexto, foi fundamental o conhecimento prévio das características da ameixa não processada (em fresco) e das transformações bioquímicas, químicas e físicas que ocorrem durante a confitagem e que conferem ao fruto confitado as características organolépticas peculiares. Este trabalho de Doutoramento pretendeu caracterizar ao nível físico-químico este produto e conhecer as alterações que ocorrem com o processamento, mas também contribuir para o conhecimento da composição química e propriedades físicas da ameixa, uma vez que o conhecimento acerca deste fruto é muito reduzido e este conhecimento é fundamental para compreender as alterações que ocorrem durante o processamento.

A textura é uma das características sensoriais mais importantes para se obter um produto confitado de boa qualidade. A textura dos frutos é influenciada pela estrutura e

composição das paredes celulares dos tecidos. Revelou-se assim de primordial importância para este trabalho de Doutoramento o estudo dos polissacarídeos da parede celular da polpa da ameixa, dado que as suas características são determinantes para a estrutura da parede e consequentemente para a textura da polpa do fruto. No decorrer do trabalho experimental foi desenvolvida uma metodologia rápida e amiga do ambiente para determinar o grau de esterificação e acetilação dos polissacarídeos (Capítulo IV), visto estas serem características importantes dos polissacarídeos da parede celular que podem afectar a textura dos frutos.

O grau de esterificação dos polissacarídeos está relacionado com a acção de uma enzima, a pectina metilesterase (PME). Esta enzima foi estudada com maior detalhe na ameixa não processada devido à sua importância para a textura dos frutos em fresco e processados. A PME foi purificada, caracterizada e o seu comportamento com o tratamento térmico foi avaliado (Capítulo V). A influência na actividade da PME com tratamentos de alta pressão foi também estudada por esta ser uma tecnologia com muitas vantagens no processamento de alimentos, permitindo a preservação dos alimentos mantendo a qualidade nutricional e as características organolépticas originais. Por outro lado, a alta pressão pode também promover a actividade da PME, permitindo melhorar a textura de alimentos de origem vegetal após o seu processamento.

A ameixa é um fruto muito rico em compostos fenólicos e estes são importantes para a qualidade nutricional do fruto. A composição em compostos fenólicos foi determinada na Ameixa d'Elvas em fresco e comparada com ameixas da mesma variedade mas de origem geográfica diferente e com ameixas de outras variedades e espécie (Capítulo VI). Este estudo teve como objectivo a identificação de compostos fenólicos que possam servir como marcadores químicos da Ameixas d'Elvas em fresco e que permitam identificar as possíveis características únicas das ameixas da variedade 'Rainha Cláudia Verde' produzidas e processadas na região do Alto Alentejo.

A composição volátil da ameixa em fresco tem sido muito estudada, assim como a sua alteração com os diversos tipos de processamento das ameixas. No entanto, era importante conhecer a composição volátil da Ameixa d'Elvas confitada para tentar explicar o seu aroma peculiar muito apreciado pelos consumidores. Os compostos voláteis da Ameixa d'Elvas confitada foram isolados, identificados e quantificados com vista a

perceber melhor quais os compostos que poderão ser responsáveis pelo seu aroma característico (Capítulo VII).

As alterações físico-químicas que ocorrem durante a confitagem da Ameixa d'Elvas foram estudadas em ameixas provenientes de dois pomares, o pomar de Vila Viçosa, que tende a originar um produto confitado de boa qualidade e o pomar do Cano, que tende a originar produtos de qualidade inferior. As ameixas dos dois pomares foram processadas separadamente e analisadas nas principais etapas do processamento, das quais resultam ameixas fervidas e confitadas. A organização celular, a textura e a composição em polissacarídeos da parede celular foram assim estudados nas ameixas em fresco, fervidas e confitadas dos dois pomares (Capítulos VIII e IX). A actividade das três principais enzimas da parede celular dos frutos relacionadas com a degradação dos polissacarídeos e com a textura dos tecidos, a pectina metilesterase (PME), a poligalacturonase (PG) e a celulase (Cel), foi quantificada nas ameixas em fresco (Capítulo IX). Este estudo teve como objectivo principal verificar se as diferenças observadas na textura dos frutos de Vila Viçosa e do Cano após a confitagem poderiam estar relacionadas com diferentes níveis de actividade destas enzimas.

No desenrolar do trabalho revelou-se importante estudar o efeito do amadurecimento na confitagem dos frutos, de modo a relacionar o estado de maturação com a qualidade do produto final. A microestrutura e a textura foram estudadas ao longo do processamento para dois estados de maturação nas ameixas de Vila Viçosa e do Cano. A evolução da actividade das enzimas da parede celular (PME, PG e Cel) ao longo da maturação das ameixas foi quantificada nas ameixas de Vila Viçosa (Capítulo X). Este estudo permitiu explicar as diferenças encontradas nas características físico-químicas das ameixas dos dois pomares. Como a sazonalidade pode influenciar as características da ameixa fresca, foi também importante estudar ameixas de anos diferentes, permitindo confirmar os resultados obtidos na colheita anterior.

Este trabalho de Doutoramento permitiu conhecer com maior profundidade as características da Ameixa d'Elvas em fresco e identificar as alterações que ocorrem com a confitagem, contribuindo para a compreensão das diferenças entre os dois pomares estudados, Vila Viçosa e Cano. Como objectivo final do trabalho foi desenvolvida uma metodologia rápida e fiável que permitisse determinar a altura ideal para colher as ameixas

em todos os pomares da região DOP, uma vez que os métodos convencionais de avaliação da maturação não se revelavam eficientes. Uma metodologia rápida e fiável com base na composição em polissacarídeos da parede celular foi proposta para determinar a altura ideal para colher as ameixas de modo a obter uma Ameixa d'Elvas confitada com a qualidade desejada, particularmente ao nível da textura (Capítulo XI).

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CAPÍTULO II

1. COMPOSIÇÃO QUÍMICA DA AMEIXA

A composição química da ameixa é apresentada na Tabela 2. Esta composição varia com a variedade da ameixa, o estado de maturação, o local onde foi produzida e as condições climatéricas.

Tabela 2 – Composição química da ameixa (adaptado de Bhutani e Joshi, 1995).

Composto	***Variação***
Peso fresco (g)	10-126
Água (g/100 g)	86-88
Hidratos de carbono (g/100 g)	6,7-9,9
Glucose	1,7-5,2
Sacarose	1,0-4,2
Frutose	0,9-3,4
Pectinas	0,8-1,0
Fibras	1,3-2,4
Proteína (g/100 g)	0,4-0,8
Cinzas (g/100 g)	0,3
Gordura (g/100 g)	0,1
Compostos fenólicos (mg/100 g)	
Antocianinas	926
Flavanóis	46-57
Minerais (mg/100 g)	
Potássio	120-190
Cálcio	6-8
Magnésio	4-7
Sódio	0-3
Ferro	0,1-0,4
Zinco	0,1
Vitaminas (mg/100 g)	
Vitamina C	4-11
Caroteno	0,3-0,8
Niacina	0,2-0,9
Vitamina E	0,7
Vitamina A	0,2
Vitamina B	0,08
Tiamina	0,02-0,05
Riboflavina	0,04-0,05
Ácidos orgânicos (meq H^+/100 g)	1,4-2,7
Ácido málico	0,1-2,5
Ácido quínico	0,12-0,41
Ácido cítrico	0,03-0,04
Acidez titulável (meq H^+/100 g)	16,3-30,5
Energia (kJ)	**125-187**

A água é o principal constituinte da polpa das ameixas, representando mais de 85% do seu peso. Os compostos maioritários na ameixa são os hidratos de carbono, que representam cerca de 10% do peso da ameixa. Neste grupo de compostos estão incluídos os açúcares solúveis, principalmente a sacarose, a glucose e a frutose, e os polissacarídeos constituintes das paredes celulares da ameixa. A composição em hidratos de carbono da ameixa é importante não só para o sabor mas também para a textura dos frutos. Os compostos fenólicos representam apenas cerca de 1% da ameixa, mas são compostos relevantes para as características sensoriais do fruto e também para as qualidades nutricionais pois possuem propriedades benéficas para a saúde, tal como será referido mais detalhadamente no capítulo 1.2.

As proteínas, os minerais e as vitaminas são compostos minoritários na ameixa, estando presentes quantidades inferiores a 1% (Tabela 2). No entanto, estes compostos têm importância a nível nutricional. Os principais aminoácidos presentes são a asparagina, o ácido aspártico, o ácido glutâmico, a glutamina, a serina, a treonina, a alanina, a valina, a leucina, a prolina e vestígios de hidroxiprolina. A ameixa é uma boa fonte de minerais como o potássio, o cálcio, o magnésio, o sódio, o ferro e o zinco. Entre todas as vitaminas presentes na polpa da ameixa (Tabela 2), a vitamina C é a única presente em quantidades significativas para ter importância a nível nutricional. O ácido málico e o ácido quínico são os ácidos orgânicos maioritários na ameixa e contribuem para o sabor ácido do fruto (Bhutani e Joshi, 1995; Ertekin *et al.*, 2006).

1.1. Polissacarídeos da parede celular

Na ameixa, o conhecimento sobre os polissacarídeos da parede celular é muito reduzido, apesar da sua importância a nível estrutural. Apenas foram estudados os polissacarídeos pécticos extraídos com água quente, que representam 29% do material insolúvel em etanol e apresentam uma composição em açúcares, em mol% de 43 de ácido D-galacturónico, 15 de galactose, 6 de arabinose, 4 de glucose, 2 de ramnose e 1 de manose (Stephen, 1995).

Os polissacarídeos dos frutos são um complexo grupo de compostos, que diferem nas suas propriedades físicas, nutricionais e funcionais, que vão sofrendo alterações ao longo do crescimento e maturação do fruto. Os polissacarídeos são os principais componentes das paredes celulares dos tecidos do parênquima.

1.1.1. Estrutura celular

As paredes celulares primárias são os principais componentes estruturais das células do parênquima dos frutos, constituídas basicamente por uma matriz composta por fibras de celulose, polissacarídeos hemicelulósicos e pécticos e pequenas quantidades de glicoproteínas, compostos fenólicos e enzimas. A união entre duas células vizinhas é denominada de lamela média e é maioritariamente constituída por polissacarídeos pécticos. A célula é também constituída pelo protoplasma contendo o citoplasma da célula, onde estão localizados o núcleo, as mitocôndrias, o vacúolo celular e outros organelos (Figura 8).

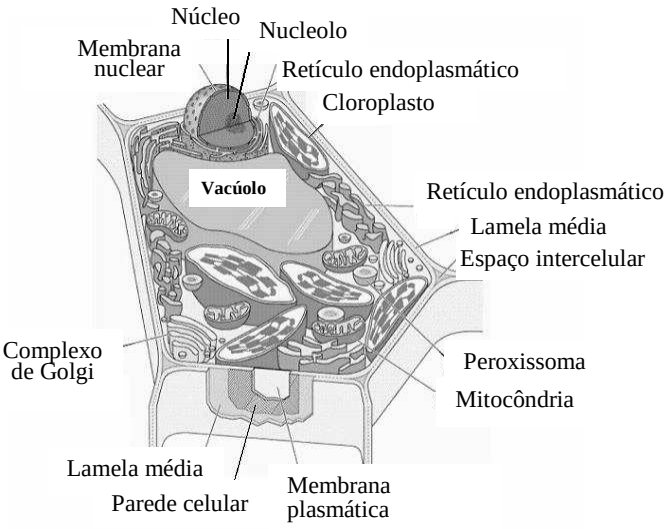


Figura 8 – Diagrama de uma célula (adaptado de www.cientic.com/imagens).

A celulose confere rigidez e resistência às paredes celulares, enquanto que os polissacarídeos pécticos e hemicelulósicos conferem alguma plasticidade e elasticidade

(Andersson *et al.*, 2006). As interacções entre todos os polímeros presentes na parede celular para formarem a matriz ainda não estão completamente elucidadas. Contudo, modelos estruturais da parede celular primária foram propostos baseados na estrutura química de cada polímero e por observação directa das paredes celulares com técnicas microscópicas avançadas (Cosgrove, 2000). Três modelos estruturais foram propostos, que diferem na interacção entre os diferentes polissacarídeos constituintes da parede celular e na sua organização espacial, assumindo que as microfibrilhas de celulose (moléculas de celulose alinhadas paralelamente) estão dispostas por camadas. No primeiro, as microfibrilhas de celulose da mesma camada e entre camadas estão ligadas entre si através dos polissacarídeos hemicelulósicos, nomeadamente xiloglucanas e xilanas de cadeias longas (Figura 9a). Neste modelo os polissacarídeos hemicelulósicos conferem resistência à parede celular e são importantes na sua integridade. No segundo modelo, as microfibrilhas de celulose estão rodeadas por polissacarídeos hemicelulósicos ligados à sua superfície, que também estão envolvidos por outros polissacarídeos associados entre si por ligações mais fracas, não existindo uma interligação directa entre as microfibrilhas (Figura 9b). No terceiro modelo, a parede celular é constituída por camadas alternadas de uma matriz constituída pela celulose e pelos polissacarídeos hemicelulósicos e de uma matriz de polissacarídeos pécticos, em que os polissacarídeos hemicelulósicos interligam as microfibrilhas de celulose da mesma camada (Figura 9c). Nestes três modelos os polissacarídeos pécticos preenchem os espaços vazios da estrutura formada pela celulose e os polissacarídeos hemicelulósicos (Cosgrove, 2000).

No entanto, os polissacarídeos pécticos parecem ser muito mais importantes para a estrutura da parede celular do que apenas o preenchimento dos espaços livres da matriz. Recentemente foi proposto outro modelo de parede celular em que a estrutura formada pelas microfibrilhas de celulose e pelos polissacarídeos hemicelulósicos está envolvida por uma matriz de polissacarídeos pécticos interligados, em que os polissacarídeos pécticos estão ligados à celulose e/ou aos polissacarídeos hemicelulósicos. A grande diferença deste modelo em relação aos outros anteriormente descritos é a de que os polissacarídeos pécticos da lamela média e da parede celular primária formam uma matriz que, na ausência virtual da celulose e dos polissacarídeos hemicelulósicos, originam uma parede celular com uma espessura e porosidade normal, apesar de terem uma resistência reduzida (Vincken *et al.*, 2003).

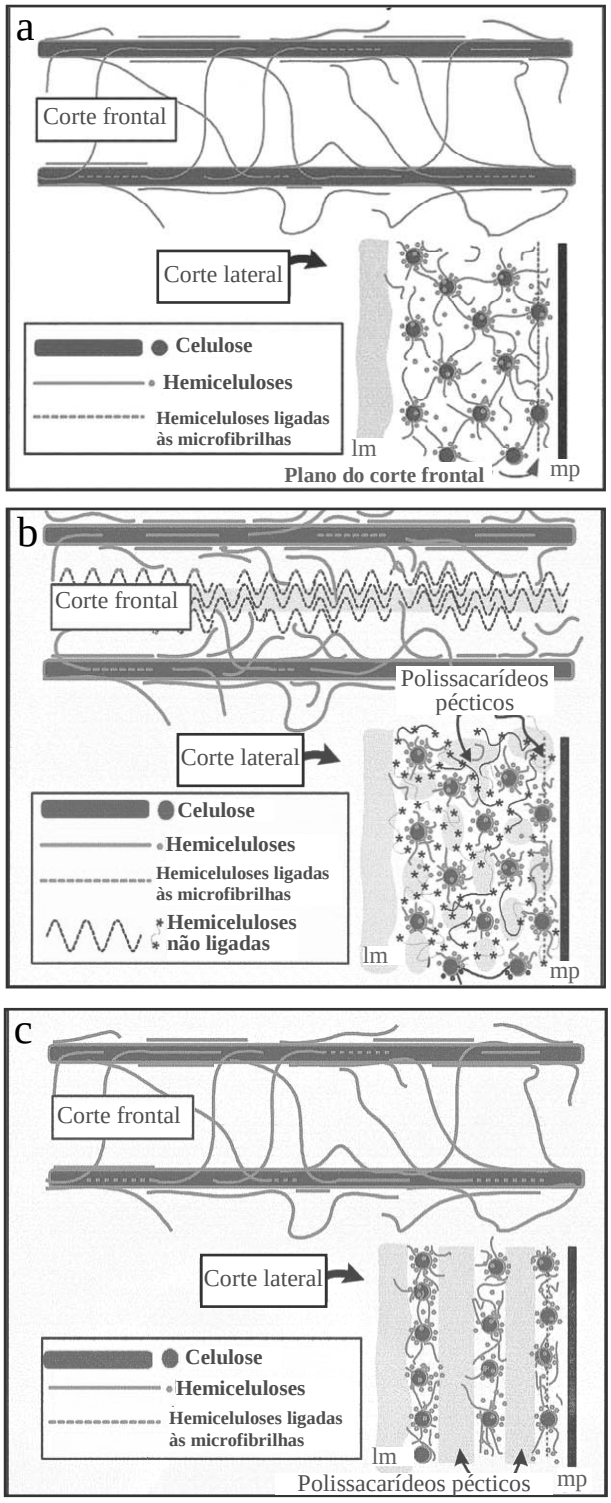


Figura 9 – Modelos estruturais da parede celular primária (lm – lamela média; mp – membrana plasmática) (Cosgrove, 2000).

Nos tecidos da polpa dos frutos podem existir células especializadas em que ocorreu lenhificação, tornando a parede celular muito mais espessa devido principalmente à

deposição de lenhina. A lenhificação tem início na região da parede celular primária e estende-se quer para o exterior, em direcção à lamela média, quer para o interior, onde se desenvolve a parede celular secundária. A deposição de lenhina é normalmente seguida de morte celular e do desaparecimento dos constituintes citoplasmáticos. As células com parede celular secundária podem ser muito alongadas (fibras) e terem funções de suporte e transporte de nutrientes, como as células dos feixes vasculares. A parede celular secundária é constituída maioritariamente por celulose, polissacarídeos hemicelulósicos e lenhina (Van Buren, 1979; Andersson *et al.*, 2006), mas continuando a possuir pequenas quantidades de polissacarídeos pécticos (Rocha *et al.*, 2000).

Os polissacarídeos da parede celular, a sua estrutura, organização e alteração com os processos fisiológicos têm sido muito estudados em vários frutos, como azeitona (Mafra *et al.*, 2001; Mafra *et al.*, 2006b), cereja (Barrett e Gonzalez, 1994), damasco (Femenia *et al.*, 1998b; a), kiwi (Redgwell *et al.*, 1991; Redgwell *et al.*, 1992; Fischer *et al.*, 1996), maçã (Johnston *et al.*, 2002), manga (Prasanna *et al.*, 2003; Yashoda *et al.*, 2005), morango (Koh e Melton, 2002; Rosli *et al.*, 2004), pêra (Martincabrejas *et al.*, 1994; Ferreira *et al.*, 2001) e pêssego (Dawson *et al.*, 1992; Heyes e Sealey, 1996; Brummell *et al.*, 2004a).

1.1.2. Polissacarídeos pécticos

Os polissacarídeos pécticos estão presentes em elevadas quantidades nos frutos e são importantes para as características dos frutos durante o seu crescimento, amadurecimento e processamento. Os polissacarídeos pécticos têm muitas funções nas paredes celulares dos tecidos a nível fisiológico relacionadas com o crescimento, o tamanho e forma da célula, a integridade e rigidez dos tecidos, transporte de iões e mecanismos de defesa contra as infecções (Andersson *et al.*, 2006). Estes polissacarídeos são depositados nas primeiras fases do crescimento dos frutos quando ocorre o aumento da área das células (Stephen, 1995).

Os polissacarídeos pécticos são uma mistura complexa de polímeros com diferentes características estruturais, devido aos diferentes graus de polimerização, esterificação e acetilação e à quantidade de açúcares neutros das cadeias laterais. Os polissacarídeos pécticos são polímeros constituídos por ácido galacturónico que se encontra

metilesterificado, denominados de pectinas, polímeros em que o ácido galacturónico se encontra desesterificado (ácidos pécticos), os sais de ácidos pécticos (pectatos) e os polissacarídeos neutros que se encontram geralmente em associação com a cadeia de ácido galacturónico. Na parede celular podem existir quatro tipos de polissacarídeos pécticos: as homogalacturonanas, as ramnogalacturonanas do tipo I (RG-I), as xilogalacturonanas e as ramnogalacturonanas do tipo II (RG-II).

As homogalacturonanas são polissacarídeos constituídos por cadeias lineares de ácido D-galacturónico em ligação α-(1→4), parcialmente esterificados (Figura 10). Alguns resíduos de ramnose em ligação α-(1→2) podem aparecer na cadeia de ácido D-galacturónico, sendo o conteúdo em ramnose de 1 a 4%. A presença de ramnose na cadeia origina irregularidades na estrutura, conferindo alguma flexibilidade (Vincken *et al.*, 2003; Andersson *et al.*, 2006).

As RG-I são polissacarídeos constituídos por ácido D-galacturónico em ligação α-(1→4) alternando com resíduos de L-ramnose em ligação α-(1→2). Cerca de 50% dos resíduos de ramnose são ramificados no carbono 4 por cadeias laterais curtas ricas em arabinose e/ou galactose (Figura 10). Estas cadeias laterais estão presentes principalmente como arabinanas, galactanas e arabinogalactanas do tipo I. A D-xilose, a D-glucose, a D-manose, a L-fucose e o ácido D-glucurónico são açúcares menos frequentes. As arabinanas são constituídas por uma cadeia principal de resíduos de L-arabinose em ligação α-(1→5) com outros resíduos de L-arabinose ligados ao carbono 2 e/ou 3. As galactanas são polissacarídeos lineares constituídos por D-galactose em ligação β-(1→4). As arabinogalactanas são polímeros com uma cadeia principal linear de resíduos de D-galactose em ligação β-(1→4) com ramificações no carbono 3 de cadeias curtas de resíduos de arabinose (20 a 40%) em ligação α-(1→5) (Figura 10).

As xilogalacturonanas são polissacarídeos pécticos ramificados em que resíduos de D-xilose podem aparecer como ramificação ligados ao carbono 3 do ácido D-galacturónico da cadeia principal (Figura 10), em que o grau de ramificação pode variar de 25 a 75%. Os resíduos de ácido galacturónico podem estar esterificados como nas cadeias de homogalacturonanas (Vincken *et al.*, 2003).

Homogalacturonanas:

→4)-α-D-Gal*p*A-(1→4)-α-D-Gal*p*A-(1→4)-α-D-Gal*p*A-(1→4)-α-D-Gal*p*A-(1→

Ramnogalacturonanas I:

→4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→

Arabinanas:

α-L-Ara*f* α-L-Ara*f*
1 1
↓ ↓
3 2
→5)-α-L-Ara*f*-(1→5)-α-L-Ara*f*-(1→5)-α-L-Ara*f*-(1→5)-α-L-Ara*f*-(1→

Galactanas:

→4)-β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→

Arabinogalactanas I:

α-L-Ara*f* α-L-Ara*f*
1 1
↓ ↓
5 5
α-L-Ara*f* α-L-Ara*f*
1 1
↓ ↓
3 3
→4)-β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→

Xilogalacturonanas:

β-D-Xyl*p* β-D-Xyl*p*
1 1
↓ ↓
3 3
→4)-α-**D**-Gal*p*A-(1→4)-α-**D**-Gal*p*A-(1→4)-α-**D**-Gal*p*A-(1→4)-α-**D**-Gal*p*A-(1→

Figura 10 – Estruturas das homogalacturonanas, ramnogalacturonanas I, xilogalacturonanas e cadeias de polissacarídeos neutros dos polissacarídeos pécticos.

A estrutura das RG-II é diferente das RG-I, pois os resíduos de ramnose são menos abundantes na cadeia principal, estando presentes maioritariamente nas cadeias laterais. As ramificações surgem nos carbonos 2 ou 3 de resíduos de ácido galacturónico. As RG-II são polissacarídeos muito ramificados com cadeias laterais complexas, podendo conter 12 açúcares diferentes, tais como apiose (Api), 2-*O*-metil-α-L-fucose, 2-*O*-metil-α-D-xilose, ácido acérico (Ace), ácido 3-desoxi-D-*mano*-2-octulosónico (KDO) e ácido 3-desoxi-D-*lixo*-2-heptulosárico (DHA) (Figura 11). Estas ramnogalacturonanas ocorrem em pequenas percentagens nas células do parênquima, mas têm uma função importante na estrutura das paredes celulares pois contribuem para a sua consolidação (Vincken et al., 2003).

1) 2Me-α-L-Fuc*p*
1
↓
2
α-D-Gal*p*-(1→2)-α-L-Ace*f*-(1→3)-β-L-Rha*p*-(1→3')-Api*f*-(1→2)-α-D-Gal*p*A
4
↑
1
α-L-Ara*p*
2
↑
1
α-L-Rha*p*-(2←1)-β-L-Ara*f*

2) α-L-Rhap-(1→5)-D-KDO*p*-(1→3)-α-D-Gal*p*A

3) β-L-Ara*f*-(1→5)-D-DHA*p*-(1→3)-α-D-Gal*p*A

4) α-D-Gal*p* 2Me-α-D-Xyl*p* β-D-Gal*p*A
1 1 1
↓ ↓ ↓
2 3 3
β-D-Glc*p*A-(1→4)-α-L-Fuc*p*-(1→4)-β-L-Rha*p*-(1→3')-Api*f*-(1→2)-α-D-Gal*p*A
2
↑
1
α-D-Gal*p*A

Figura 11 – Estruturas das cadeias laterais da ramnogalacturonana II**.**

A organização dos polissacarídeos pécticos na parede celular ainda não está completamente elucidada. As homogalacturonanas e as RG-I parecem formar uma extensa cadeia principal com uma distribuição intramolecular bem definida, em que regiões muito ramificadas com açúcares neutros estão separadas por zonas de cadeia principal sem ramificações, contendo quase exclusivamente resíduos de ácido galacturónico (Stephen, 1995; Andersson *et al.*, 2006). No entanto, um modelo estrutural alternativo foi proposto de acordo com alguns estudos mais recentes, em que a principal diferença reside na cadeia principal dos polissacarídeos pécticos ser constituída apenas por RG-I e as homogalacturonanas serem ramificações da RG-I. Neste modelo os polissacarídeos pécticos podem ter cadeias laterais apenas de um tipo ou vários tipos de cadeias laterais distribuídos de forma aleatória na cadeia principal ou agrupadas consoante o tipo de polissacarídeos (Vincken *et al.*, 2003).

Os resíduos de ácido galacturónico dos polissacarídeos pécticos podem estar esterificados com metanol em *C*-6 e/ou ácido acético em *O*-2 e *O*-3. O grau de metilação é definido como a percentagem de grupos carboxílicos esterificados com metanol e o grau de acetilação é definido como a percentagem de resíduos de ácido galacturónico esterificados com ácido acético. Normalmente as pectinas de frutos têm elevados graus de metilação e baixos graus de acetilação (Stephen, 1995; Prasanna *et al.*, 2007). Esta é uma característica estrutural dos polissacarídeos pécticos muito importante associada com a textura dado que a diminuição do grau de esterificação está directamente correlacionado com a perda de textura dos frutos (Chatjigakis *et al.*, 1998).

Os polissacarídeos pécticos podem também estar esterificados, no terminal não redutor dos resíduos de arabinose e galactose, com os ácidos ferúlico e cumárico. Estes substituintes facilitam as interacções entre as moléculas de polissacarídeos pécticos com outros polissacarídeos presentes na parede celular, sendo importantes para a estrutura da parede celular (Prasanna *et al.*, 2007).

A lamela média é constituída por polissacarídeos pécticos estruturalmente diferentes dos que constituem a parede celular primária. Os polissacarídeos da lamela média são capazes de se associarem por intermédio de catiões, como o cálcio, por isso são polímeros com zonas constituídas por resíduos de ácido galacturónico não esterificados intercaladas por zonas com polímeros pouco ramificados com cadeias laterais curtas e com elevado grau de esterificação. Pelo contrário, as pectinas da parede celular primária são muito ramificadas e com cadeias laterais mais longas, isto é, com um elevado conteúdo em açúcares neutros (Selvendran, 1985; Prasanna *et al.*, 2007).

Os polissacarídeos pécticos têm capacidade de formar géis na presença de sacarose em meios ligeiramente ácidos, pois a sacarose diminui a actividade da água, promovendo a interacção entre as cadeias e o meio ácido diminui as cargas negativas dos grupos carboxílicos diminuindo a repulsão electrostática entre as cadeias dos polissacarídeos. Estes géis são estabilizados por ligações de hidrogénio que se formam entre as cadeias de ácido galacturónico e por ligações hidrofóbicas entre os grupos metilo dessas cadeias. A formação do gel é favorecida pela elevada percentagem de esterificação, baixa percentagem de acetilação e pela presença de cadeias longas pouco ramificadas. Os polissacarídeos pécticos com baixo grau de esterificação também podem gelificar por

interacção entre os grupos carboxílicos e os catiões, como o cálcio, se presentes no meio. A capacidade de gelificação dos polissacarídeos pécticos é uma propriedade importante para a textura dos frutos, pois contribui para a manutenção da integridade dos tecidos ao longo da maturação e processamento.

1.1.3. Celulose

A celulose é um dos principais constituintes da parede celular primária, presente na forma de microfibrilhas. Este polissacarídeo é composto por cadeias lineares de glucose com ligações β-(1→4) (Figura 12). As cadeias estão associadas entre si por ligações de hidrogénio intra e intermoleculares formando microfibrilhas. A estrutura organizada da celulose torna-a num polímero de pouca solubilidade (Andersson *et al.*, 2006). Durante o amadurecimento dos frutos ocorre alguma degradação das microfibrilhas de celulose por acção da celulase. A celulase caracteriza-se por ter várias actividades enzimáticas capazes de degradar a celulose em derivados solúveis e a sua actividade contribui para a perda de textura dos frutos com o amadurecimento (Prasanna *et al.*, 2007).

→4)-β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→

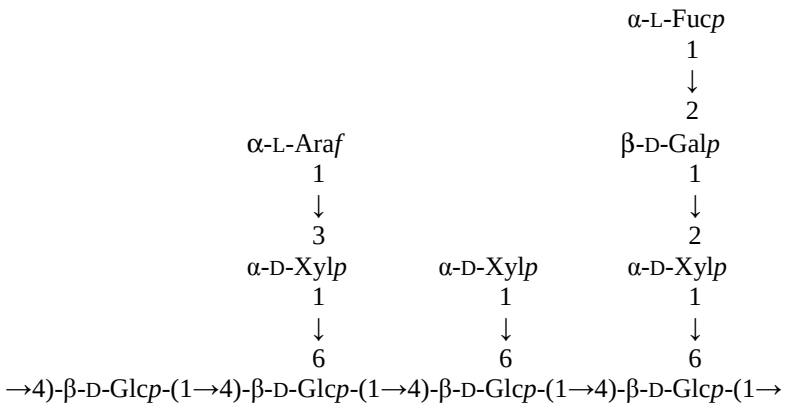
Figura 12 – Estrutura da celulose.

1.1.4. Polissacarídeos hemicelulósicos

As hemiceluloses da parede celular primária dos frutos são um grupo de polissacarídeos composto principalmente por xiloglucanas, mas também por xilanas, glucomananas e arabinogalactanas do tipo II. As xiloglucanas estão normalmente presentes na parede celular primária associadas às microfibrilhas de celulose por pontes de hidrogénio, podendo representar entre 20 a 25% dos polímeros. Este polissacarídeo tem uma cadeia principal de resíduos de glucose em ligação β-(1→4) com cadeias laterais

constituídas por resíduos de α-D-xilose ligados ao carbono 6 de alguns resíduos de glucose e por β-D-galactose ligada ao carbono 2 ou α-L-arabinose no carbono 3 dos resíduos de xilose. A proporção de glucose, xilose e galactose neste polissacarídeo é de 4:3:1. Resíduos de α-L-fucose podem estar ligados ao carbono 2 da galactose (Figura 13a). Alguns resíduos de galactose da xiloglucana podem estar acetilados (Andersson *et al.*, 2006).

a)



b)

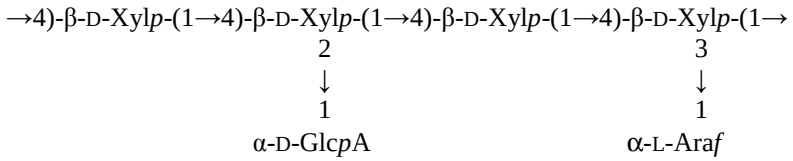


Figura 13 - Estruturas dos principais polissacarídeos hemicelulósicos: a) xiloglucana e b) xilana.

As xilanas estão presentes principalmente nos tecidos lenhificados e são constituídas por uma cadeia principal de xilose em ligação β-(1→4) com cadeias laterais curtas compostas maioritariamente por resíduos de ácido glucurónico e também alguma arabinose (Figura 13b). Estes polímeros podem ter cerca de 10% dos resíduos de xilose ramificados e 5 a 17% dos resíduos acetilados.

As glucomananas são polissacarídeos com uma cadeia principal composta por D-glucose e D-manose em ligação β-(1→4) com ramificações ocasionais de resíduos únicos de α-D-galactose nos resíduos de D-manose. Estas hemiceluloses também estão ligadas por pontes de hidrogénio às microfibrilhas de celulose da matriz da parede celular (Selvendran, 1985; Andersson *et al.*, 2006).

1.2. Compostos fenólicos

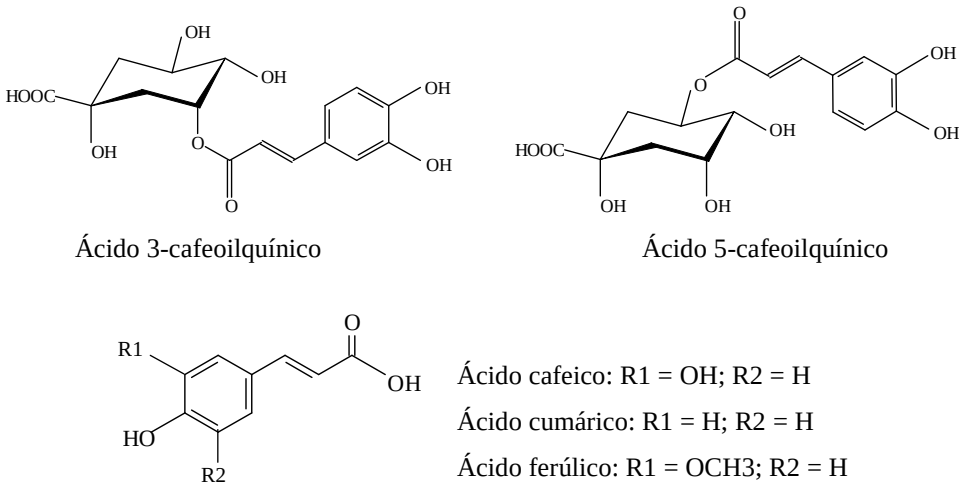
Os compostos fenólicos são responsáveis por algumas características organolépticas da ameixa, nomeadamente a sua cor, sabor e aroma. Numa fase inicial do amadurecimento, a presença de compostos fenólicos em concentrações mais elevadas é responsável pelo sabor adstringente e ácido do fruto (Cheynier, 2005). Estes compostos estão localizados principalmente na parede celular, associados aos polissacarídeos, e também nos vacúolos, onde são armazenados os compostos fenólicos solúveis e os seus derivados (Cheynier, 2005). Os compostos fenólicos ligados aos polissacarídeos podem desempenhar uma função estrutural importante nas paredes celulares, alterando as suas propriedades mecânicas. Por exemplo, as ligações entre os polissacarídeos da parede celular e os dímeros de ácido ferúlico conferem resistência aos tecidos, mantendo a adesão intercelular (Waldron *et al.*, 1997).

As propriedades biológicas dos compostos fenólicos podem ser diversas, estando relacionadas com o número e acessibilidade dos grupos fenol destes compostos. Entre as propriedades biológicas mais importantes estão as suas actividades antioxidante, anti-inflamatória, anti-tumoral e anti-mutagénica. A capacidade antioxidante é uma característica importante, pois pode ser benéfica para a saúde, nomeadamente em doenças crónicas e degenerativas. Os compostos fenólicos são preferencialmente oxidados protegendo a célula contra a oxidação por acção dos radicais livres de alguns componentes da célula, como lípidos, proteínas e ácidos nucleicos (Tomás-Barberán e Espin, 2001; Kim *et al.*, 2003c; Cheynier, 2005).

O conteúdo e composição em compostos fenólicos das ameixas varia com a variedade, condições de cultivo, condições climatéricas, origem geográfica e estado de maturação dos frutos (Tomás-Barberán e Espin, 2001; Imeh e Khokhar, 2002; Kim *et al.*, 2003b; Lombardi-Boccia *et al.*, 2004; Rupasinghe *et al.*, 2006). As ameixas têm um elevado conteúdo em compostos fenólicos com elevada actividade antioxidante, quando comparadas com outros frutos (Wang *et al.*, 1996; Tomás-Barberán *et al.*, 2001; Gil *et al.*, 2002; Imeh e Khokhar, 2002; Kim *et al.*, 2003a,c). A acumulação de compostos fenólicos

é maior na epiderme da ameixa do que na polpa (Tomás-Barberán *et al.*, 2001; Cevallos-Casals *et al.*, 2006).

Os compostos fenólicos maioritários na ameixa pertencem a três classes: ácidos hidroxicinâmicos, flavanóis e flavonóis. Na classe dos ácidos hidroxicinâmicos, os principais compostos presentes na polpa da ameixa são os ácidos 3-cafeoilquínico (ácido neoclorogénico) e 5-cafeoilquínico (ácido clorogénico) (Figura 14). Em menores concentrações foram também detectados os ácidos cafeico, cumárico e ferúlico (Tomás-Barberán *et al.*, 2001; Fang *et al.*, 2002; Kim *et al.*, 2003b). Estes ácidos fenólicos são moléculas simples sendo a principal fonte de actividade antioxidante das ameixas, com actividades superiores à vitamina C e aos carotenóides (Chun *et al.*, 2003; Piga *et al.*, 2003; Chun e Kim, 2004).



Ácido 3-cafeoilquínico

Ácido 5-cafeoilquínico

Ácido cafeico: R1 = OH; R2 = H

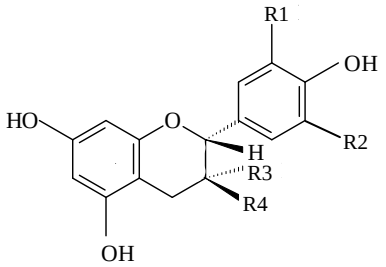
Ácido cumárico: R1 = H; R2 = H

Ácido ferúlico: R1 = OCH3; R2 = H

Figura 14 – Estrutura dos principais ácidos hidroxicinâmicos da ameixa.

Os flavonóides são um grupo de compostos fenólicos constituído por compostos que possuem um núcleo comum com 2 anéis fenólicos e 1 anel heterocíclico. Estes estão divididos em vários grupos que diferem no estado de oxidação do anel heterocíclico, como os flavanóis, os flavonóis e as antocianinas (Figura 15). Os principais flavanóis detectados na ameixa estão presentes como dímeros e trímeros de catequina e epicatequina. Procianidinas de maior peso molecular, oligómeros e polímeros, também foram detectadas na polpa da ameixa mas em pequenas quantidades (Tomás-Barberán *et al.*, 2001). Estas

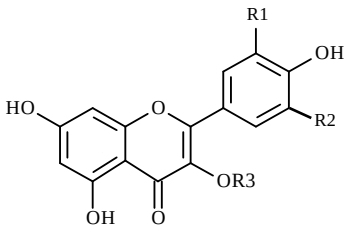
procianidinas podem diferir nas unidades que a constituem (catequina e epicatequina), na sua sequência, na posição das ligações interflavanicas (C4-C6 ou C4-C8 nas estruturas do tipo B e ligações adicionais C2-*O*-C7 ou C2-*O*-C5 nas do tipo A) e no tamanho da cadeia (Tomás-Barberán *et al.*, 2001; Pascual-Teresa *et al.*, 2000).



Flavanóis

Catequina: R1 = OH; R2 = H; R3 = H; R4 = OH

Epicatequina: R1 = OH; R2 = H; R3 = OH; R4 = H

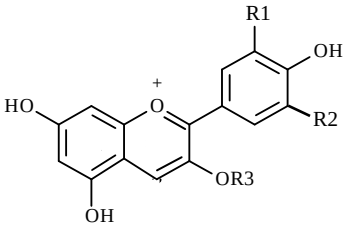


Flavonóis

Rutina: R1 = OH; R2 = H; R3 = rutinosídeo

Quercitina: R1 = OH; R2 = H; R3 = H

Campferol: R1 = H; R2 = H; R3 = H



Antocianinas

Cianidina: R1 = OH; R2 = H; R3 = glicosídeo

Peonidina: R1 = OCH_3; R2 = H; R3 = glicosídeo

Figura 15 – Estrutura dos principais compostos fenólicos da ameixa pertencentes às classes dos flavonóides.

Os flavonóis estão presentes apenas na epiderme da ameixa, sendo a rutina o principal flavonol. Outros compostos derivados da quercitina e também do campferol (Figura 15) com ligações a diferentes açúcares, como glucose, galactose, arabinose, ramnose e xilose, podem ocorrer mas em pequenas quantidades (Tomás-Barberán *et al.*, 2001; Piga *et al.*, 2003). Para além dos ácidos clorogénicos, os flavonóis são também uma

importante fonte de actividade antioxidante das ameixas (Chun *et al.*, 2003; Piga *et al.*, 2003; Chun e Kim, 2004).

Um elevado número de outros flavonóides foram identificados na ameixa, em concentrações reduzidas, devido às numerosas substituições que podem ocorrer nestas moléculas, nomeadamente com grupos hidroxílicos, metoxílicos ou glicosídicos, que por sua vez podem também estar substituídos (resíduos glicosídicos e grupos acetilo).

As antocianinas são os principais compostos responsáveis pela cor das ameixas (vermelha e púrpura), juntamente com os carotenóides, estando localizadas apenas na epiderme dos frutos (Eskin, 1990). A cianidina-3-rutinosídeo, a cianidina-3-glucosídeo e a peonidina-3-rutinosídeo são as antocianinas maioritárias presentes na epiderme da ameixa (Figura 15) (Tomás-Barberán *et al.*, 2001; Kim *et al.*, 2003b; Piga *et al.*, 2003).

1.3. Compostos voláteis

Os compostos voláteis são compostos com baixo ponto de ebulição que estão presentes na fase gasosa que rodeia a ameixa. Estes compostos apesar de estarem presentes em quantidades muito reduzidas são responsáveis pelo seu aroma desde que estejam presentes em quantidades superiores ao limite de percepção sensorial, sendo assim muito importantes para as características sensoriais da ameixa.

Os ésteres são a classe de compostos qualitativamente mais importante presente nos extractos da fracção volátil das ameixas. Na generalidade estes compostos têm descritores de aroma a frutos. Numa análise quantitativa, para além dos ésteres, os álcoois ou aldeídos também podem representar uma fracção importante dos extractos, dependendo da variedade da ameixa (Crouzet *et al.*, 1990). Os compostos mais importantes que contribuem para o aroma das ameixas em geral são: o nonanal, o *cis*-3-hexenol, o linalol, a γ-decalactona, o benzaldeído, a γ-octalactona, o 1-hexanol e o 2-feniletanol (Figura 16) (Williams e Ismail, 1981; Crouzet *et al.*, 1990).

A composição volátil das ameixas depende da sua espécie e variedade. Nas ameixas europeias (*Prunus domestica* L.) as maiores diferenças entre as variedades ocorrem a nível quantitativo e não qualitativo. Nesta espécie de ameixas, uma maior concentração de

nonanal, γ-decalactona, benzaldeído e 2-feniletanol está relacionada positivamente com o aroma e parecem ser responsáveis pelo seu aroma característico (Williams e Ismail, 1981).

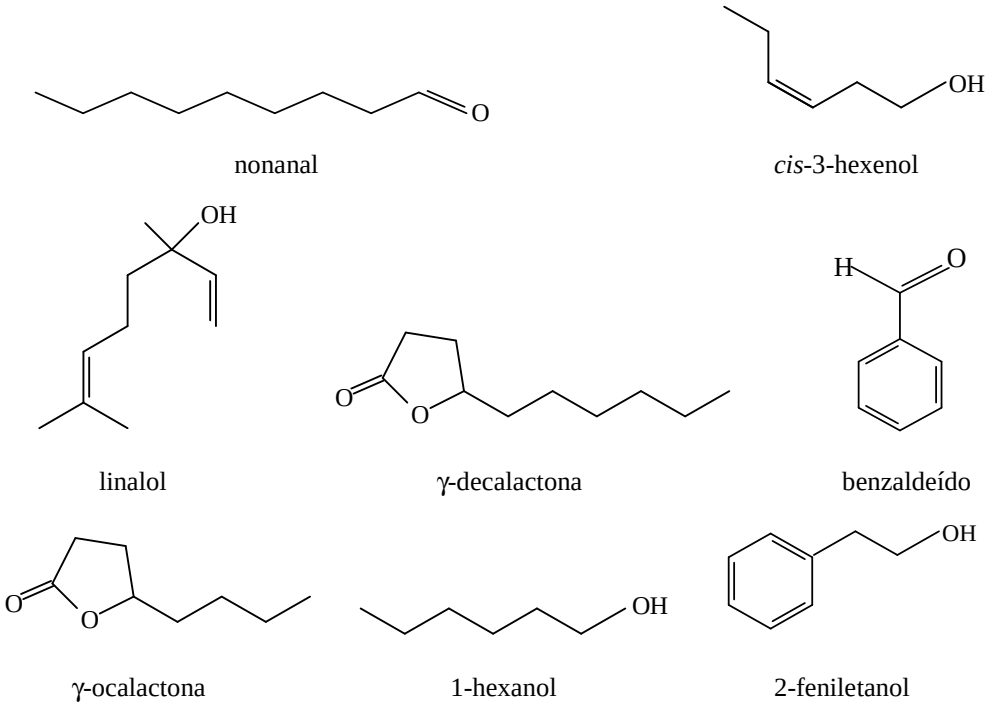


Figura 16 - Estrutura dos principais compostos voláteis que contribuem para o aroma das ameixas.

2. ALTERAÇÕES DAS CARACTERÍSTICAS FÍSICAS DA AMEIXA

A cor, a textura, o sabor e o aroma da ameixa são propriedades importantes para a qualidade final do fruto. As características organolépticas vão-se desenvolvendo ao longo do processo de maturação do fruto como resultado de modificações químicas e bioquímicas.

O processamento origina também alterações físicas nas ameixas importantes para a obtenção das propriedades organolépticas características dos diversos tipos de produtos processados. As modificações físicas que podem ocorrer nos frutos durante o seu processamento, principalmente com o tratamento térmico, estão relacionadas com a alteração da composição química.

2.1. Cor

Na epiderme ou pele da ameixa, com o avanço da maturação, a cor verde passa progressivamente para vermelho, amarelo, rosa ou preto, segundo a variedade. Esta mudança de cor está associada à maturação em muitas espécies de frutos. No entanto, nas ameixas pode não reflectir uma mudança de maturação, pois algumas variedades podem apresentar a cor definitiva sem estarem totalmente maduras. A maturação também origina mudanças na cor da polpa. As alterações na cor, ao longo da maturação das ameixas, são devidas ao aumento do conteúdo em antocianinas e carotenóides e à diminuição da clorofila (Eskin, 1990).

A cor do fruto diminui com o processamento, principalmente devido a alterações nas antocianinas, compostos muito instáveis ao tratamento térmico. As antocianinas podem ser mais estáveis se formarem complexos com outros compostos fenólicos, hidratos de carbono, aminoácidos e iões, como cálcio, magnésio e potássio (Eskin, 1990). As alterações nas antocianinas ocorrem devido à acção conjunta da temperatura e das enzimas, principalmente da polifenol oxidase e da peroxidase, enquanto não tenham sido inactivadas

pelo tratamento térmico (Piga *et al.*, 2003). A enzima polifenol oxidase catalisa duas reacções diferentes na presença do oxigénio molecular, a hidroxilação e a oxidação dos fenóis, obtendo-se quinonas. Estes compostos polimerizam facilmente originando compostos com cor castanha, preta ou vermelha, denominados de melaninas. A peroxidase catalisa a oxidação de vários compostos na presença de peróxido de hidrogénio. Estas duas enzimas actuam em coordenação pois a oxidação por acção da polifenol oxidase origina peróxido de hidrogénio (Tomás-Barberán e Espin, 2001). Para além das temperaturas elevadas, o pH baixo e a presença de oxigénio promovem a degradação das antocianinas (Eskin, 1990). Estas reacções provocam o escurecimento do fruto, o que normalmente não é desejável nos produtos processados de ameixa.

As reacções de Maillard, entre os monossacarídeos e os aminoácidos presentes nos frutos, originam pigmentos de cor amarela ou castanha, denominados de melanoidinas. Estes compostos contribuem também para a alteração da cor das ameixas com o processamento, principalmente nas ameixas secas (Davídek *et al.*, 1990).

2.2. Textura

A textura da ameixa é uma das características sensoriais importantes para o consumidor. A firmeza da polpa da ameixa diminui com o amadurecimento do fruto, tornando o fruto mais macio e sumarento. Esta alteração na textura do fruto está relacionada com modificações estruturais dos polissacarídeos que constituem a parede celular primária e a lamela média. Estas alterações incluem a despolimerização, desesterificação, solubilização e redução do tamanho dos polissacarídeos e ocorrem devido principalmente à acção combinada de diversas enzimas (Van Buren, 1979; Eskin, 1990; Edwards, 1999).

Ao longo do amadurecimento ocorre a diminuição da cadeia de ácido galacturónico, do grau de esterificação e do peso molecular dos polissacarídeos pécticos (O'Donoghue *et al.*, 1997; Redgwell *et al.*, 1997a; Redgwell *et al.*, 1997b; Femenia *et al.*, 1998a; Wakabayashi, 2000). As alterações dos polissacarídeos pécticos originam a diminuição da adesão intercelular e da resistência dos tecidos da parede celular, sendo um dos principais factores responsáveis pela modificação na textura (Eskin, 1990; Taylor *et al.*, 1995;

Wakabayashi, 2000). A degradação dos polissacarídeos pécticos é essencialmente catalisada pela acção concertada de dois tipos de enzimas, a pectina metilesterase e a poligalacturonase.

A pectina metilesterase catalisa a desesterificação dos resíduos de ácido galacturónico convertendo as pectinas de elevado grau de metilação em polissacarídeos de baixo grau de metilação. A desesterificação parcial dos resíduos de ácido galacturónico dos polissacarídeos pécticos pela PME torna-os mais susceptíveis à acção da poligalacturonase. Existem dois tipos de poligalacturonases, a endo- e a exo-poligalacturonase. A primeira catalisa a hidrólise de ligações glicosídicas adjacentes a grupos carboxílicos não metilados no interior da cadeia, provocando a despolimerização dos polissacarídeos pécticos. O aumento da actividade desta enzima com o amadurecimento é acompanhado por um aumento da solubilidade dos polissacarídeos pécticos em água. A exo-poligalacturonase catalisa a hidrólise das ligações glicosídicas do resíduo do terminal não redutor dos polissacarídeos pécticos, libertando ácido galacturónico. A acção desta enzima causa um aumento dos grupos redutores, mas não afecta significativamente a solubilidade dos polissacarídeos. A perda de textura dos frutos com o amadurecimento está directamente relacionada com a actividade dos dois tipos de poligalacturonase, uma perda extensa ocorre se estiverem presentes a endo- ou a endo- e a exo- poligalacturonase, enquanto que uma perda de textura limitada ocorre se apenas a exo- poligalacturonase estiver presente (Wakabayashi, 2000; Prasanna *et al.*, 2007).

A actividade da pectina metilesterase aumenta até uma determinada fase de maturação do fruto e depois diminui com o aumento da actividade da poligalacturonase. A acção combinada das duas enzimas é necessária para ocorrer uma extensa degradação dos polissacarídeos pécticos durante a maturação (Eskin, 1990; Wakabayashi, 2000).

A ramnogalacturonase, que catalisa a hidrólise da ligação glicosídica entre resíduos de ácido galacturónico e ramnose, pode também contribuir para a degradação dos polissacarídeos pécticos (Prasanna *et al.*, 2007). Para além destas enzimas, a presença de β-galactosidades e β-galactanases promovem também a degradação dos polissacarídeos pécticos por degradação das arabinogalactanas das cadeias laterais (Wakabayashi, 2000; Prasanna *et al.*, 2007; Vicente *et al.*, 2007; Goulão e Oliveira, 2008).

A degradação dos polissacarídeos hemicelulósicos, nomeadamente as xiloglucanas, em cooperação com a degradação dos polissacarídeos pécticos, também contribui para as alterações de textura dos frutos. Ao longo da maturação dos frutos ocorre uma diminuição do conteúdo em xiloglucanas e da sua massa molecular, indicando a degradação e despolimerização deste polissacarídeo (Redgwell *et al.*, 1991; O'Donoghue *et al.*, 1997; Wakabayashi, 2000; Mafra *et al.*, 2006b). Esta alteração das xiloglucanas ocorre devido à acção de hidrolases, nomeadamente de xiloglucanases e da celulase. A celulase é uma enzima com actividade de endo-1,4-β-glucanase, exo-1,4-β-glucanase e endo-1,4-β-glucosidase. A endo-1,4-β-glucanase degrada todos os polissacarídeos com ligações glicosídicas β-(1→4) entre resíduos de glucose no interior da cadeia, a exo-1,4-β-glucanase cliva ligações glicosídicas nos resíduos não redutores originando glucose e celobiose e a endo-1,4-β-glucosidase cliva a ligação glicosídica da celobiose. Esta enzima não é capaz de degradar a celulose cristalina. A actividade de celulase aumenta drasticamente com o amadurecimento dos frutos e está directamente relacionada com as alterações na textura (Prasanna *et al.*, 2007).

Provavelmente, a degradação das moléculas de xiloglucana no início do processo de maturação dos frutos pode contribuir, mesmo que parcialmente, para degradar os complexos com a celulose, provocando a perda da integridade da parede celular e também aumentando a difusão de outras hidrolases na parede celular, como β-galactosidades, β-galactanases, β-xilanases e β-glucanases que degradam outros polissacarídeos hemicelulósicos (Van Buren, 1979; Wakabayashi, 2000; Prasanna *et al.*, 2007; Vicente *et al.*, 2007).

O processamento térmico da ameixa induz modificações na textura dos frutos. A textura final da ameixa processada depende da textura do fruto antes de processar, pois frutos muito maduros são mais susceptíveis à degradação térmica (Edwards, 1999). A estrutura celular bem organizada do fruto fresco transforma-se numa estrutura celular amorfa após o tratamento térmico. Este tipo de processamento destrói a integridade do plasmalema e, consequentemente, a perda do turgor das células. Também ocorrem algumas alterações na parede celular, principalmente na lamela média, provocando a separação das células. Estas alterações originam diferenças no corte do fruto ao ser consumido. No fruto fresco a fractura do tecido ocorre na parede celular, libertando o conteúdo da célula. No

fruto processado termicamente, pelo contrário, a fractura ocorre ao longo da lamela média, separando completamente as células que mantêm o seu conteúdo. Por esta razão as ameixas processadas não são tão sumarentas como as não processadas (Waldron *et al.*, 1997; Edwards, 1999).

A principal alteração a nível celular observada com o processamento térmico é a perda de adesão celular por degradação da lamela média. O aquecimento provoca a degradação dos polissacarídeos pécticos da lamela média principalmente devido ao processo de β-eliminação. A degradação por β-eliminação consiste na quebra da ligação glicosídica entre dois resíduos de ácido galacturónico, com desidratação e formação de uma dupla ligação entre os carbonos 4 e 5. O resíduo sujeito à reacção de β-eliminação tem de ter o grupo carboxílico esterificado, pelo que a extensão da degradação das pectinas depende do seu grau de metilação. A presença de iões cálcio nos tecidos tem um efeito contraditório na textura dos frutos. Por um lado, aumenta consideravelmente a velocidade da reacção de β-eliminação, por outro lado, os iões cálcio formam complexos com as cadeias de pectina, aumentando a resistência destes polissacarídeos à degradação térmica (Van Buren, 1979). A presença de ácido ferúlico ligado aos polissacarídeos pécticos parece também ser importante para a estabilidade térmica da lamela média e consequentemente para a textura dos frutos (Waldron *et al.*, 1997).

Algumas enzimas descritas como intervenientes no processo de maturação dos frutos também podem ter um papel importante na degradação dos polissacarídeos da parede celular com o tratamento térmico. Algumas destas enzimas podem ser activadas a temperaturas entre os 50 e 80 ºC, como a pectina metilesterase. Outras podem ser reversivelmente inactivadas e posteriormente reactivadas quando os produtos são armazenados à temperatura ambiente. Apenas tratamentos térmicos drásticos podem inactivar completamente todas as enzimas da parede celular (Eskin, 1990; Belitz *et al.*, 2004). Este conhecimento sobre as características das enzimas permite melhorar algumas propriedades dos frutos processados. Nomeadamente, usando adequadas condições de processamento (temperatura, pressão, pH e catiões) é possível controlar a actividade da pectina metilesterase e inactivar a poligalacturonase (Alonso *et al.*, 1997).

2.3. Aroma

Os compostos voláteis são produzidos ao longo de todo o processo de crescimento e maturação dos frutos, o que determina o aroma das ameixas (Bhutani e Joshi, 1995). O conteúdo em hidrocarbonetos diminui com o amadurecimento, tornando-se esta classe de compostos pouco importante em ameixas maduras. Estes compostos parecem ser responsáveis pela diminuição do odor a fresco característico dos frutos imaturos. Os aldeídos também diminuem drasticamente com a maturação dos frutos, com excepção do nonanal. A diminuição destes compostos pode favorecer a qualidade do aroma das ameixas maduras pois a maioria está associada a um odor a verde e a pungente. Os compostos terpénicos aumentam ao longo do processo de maturação, em que o linalol é o composto mais importante para o aroma das ameixas maduras. A concentração das lactonas aumenta drasticamente com o amadurecimento das ameixas, nomeadamente a γ-octalactona e a γ-decalactona. As lactonas são um grupo de compostos importante para o aroma da ameixa madura, pois contribui para o seu odor frutado (Crouzet *et al.*, 1990).

O processamento das ameixas induz alterações na sua composição volátil e, consequentemente, no aroma do produto final. Por um lado, o processamento degrada alguns compostos voláteis da ameixa, mas por outro lado, estas alterações originam uma variada gama de outros compostos voláteis, importantes para o aroma das ameixas processadas (Crouzet *et al.*, 1990; Belitz *et al.*, 2004). Nas reacções de Maillard formam-se alguns compostos voláteis que contribuem para o aroma dos produtos de ameixa obtidos por tratamento térmico.

As ameixas secas contêm menos cetonas, álcoois, aldeídos alifáticos, hidrocarbonetos e ésteres do que as ameixas não processadas. Esta diminuição dos compostos voláteis é uma consequência do processamento a que as ameixas foram submetidas (temperatura elevada associada a ventilação). As elevadas temperaturas podem causar a degradação química de alguns compostos, nomeadamente os açúcares e os aminoácidos (diminuição de 75 a 80%), originando outros compostos que são importantes para o aroma das ameixas secas. A presença de furfural, 5-metilfurfural, 2-acetilfurano e 2-hidroximetil-furano (Figura 17) nas ameixas secas é indicativa da ocorrência desse tipo de reacções químicas. O tratamento térmico origina também a degradação dos carotenóides, dando origem, por

exemplo, à damascenona (Figura 17). Entre todos os compostos detectados, as cetonas são o grupo de compostos mais importantes para o odor a ameixa processada, principalmente a damascenona. A quantidade de lactonas aumenta com o processamento pois provêem dos ácidos hidroxílicos correspondentes. A presença da damascenona e das γ-lactonas são responsáveis por um aroma intenso e agradável, característico das ameixas processadas (Williams e Ismail, 1981; Crouzet *et al.*, 1990). A concentração de benzaldeído também aumenta nas ameixas secas, em comparação com as ameixas não processadas, sendo este composto também importante para o aroma do produto final.

Nas ameixas conservadas em lata os compostos carbonílicos são muito importantes para o aroma. Após o processamento destas ameixas, os compostos maioritários são o benzaldeído, o furfural, o 2-acetilfurano e o nonanal. O aroma das ameixas em lata foi descrito como uma mistura de odores a amêndoa e a madeira. Estes odores são devidos aos elevados conteúdos em benzaldeído e nonanal, respectivamente. A diferença no aroma das ameixas antes e após o processamento pode também ser devida à ausência de linalol, γ-octalactona e γ-decalactona nas ameixas processadas (Ismail *et al.*, 1980b).

O benzaldeído, o furfural e o nonanal foram também os principais compostos detectados na fracção volátil da compota de ameixa, à semelhança do que foi descrito para os outros tipos de processamento térmico. Alguns dos compostos provêem da degradação enzimática activada numa primeira fase do tratamento térmico e também da degradação química dos açúcares e aminoácidos. Algumas diferenças foram detectadas entre o aroma da compota e das ameixas em lata, provavelmente devido ao diferente estado de maturação das ameixas e/ou às diferenças no tempo e intensidade do tratamento térmico aplicado aos frutos nos dois tipos de processamento (Crouzet *et al.*, 1990).

Nos produtos fermentados de ameixa, os ésteres foram os compostos maioritários a nível quantitativo e qualitativo, como também foi descrito para as ameixas não processadas. Contudo, após a fermentação, os ésteres são constituídos maioritariamente por ésteres etílicos e metílicos. Estes produtos fermentados revelaram ter um odor característico a ameixa, provavelmente devido à presença de linalol, benzaldeído e γ-decalactona. Para além destes compostos, o eugenol (Figura 17) foi detectado em elevada quantidade neste produto fermentado em comparação com as ameixas em fresco. O eugenol parece formar-se durante o processo de fermentação devido à degradação

enzimática do álcool correspondente. O furfural foi também detectado em elevadas quantidades neste tipo de produtos de ameixa, contribuindo para o seu aroma. Este composto é proveniente da temperatura elevada que ocorre durante o processo de destilação da ameixa fermentada (Ismail *et al.*, 1980a).

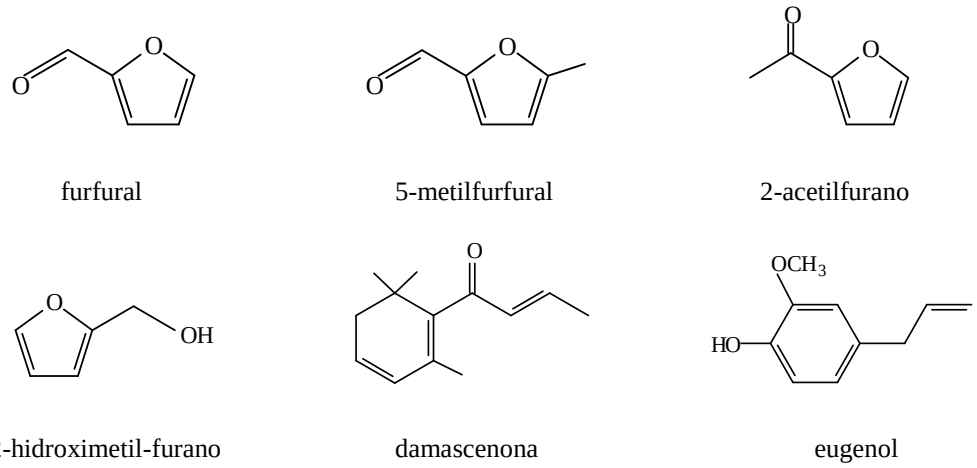


Figura 17 - Estrutura de alguns dos principais compostos voláteis formados durante os diferentes tipos de processamento das ameixas.

2.4. Sabor

Os compostos fenólicos estão principalmente associados a sabores ácidos e adstringentes. A diminuição do seu conteúdo nos frutos origina uma melhoria nas características organolépticas. O conteúdo em compostos fenólicos é elevado nas primeiras fases do desenvolvimento do fruto, diminuindo nas fases seguintes e nas últimas fases mantém-se constante. Os compostos fenólicos são muito reactivos, sendo bons substratos para várias enzimas, tais como a polifenol oxidase, a peroxidase, as glicosidases e as esterases. Por acção destas enzimas podem ocorrer alterações dos compostos fenólicos com o amadurecimento (Cheynier, 2005). O conteúdo em flavanóis diminui com o amadurecimento em algumas variedades de ameixa, contribuindo para a redução do sabor adstringente dos frutos (Tomás-Barberán *et al.*, 2001). Por outro lado, a interacção das

procianidinas com outros constituintes do fruto, nomeadamente polissacarídeos pécticos libertados durante o amadurecimento do fruto, impede a sua ligação às proteínas da saliva, diminuindo a sua adstringência (Cheynier, 2005).

O processamento dos frutos origina a degradação dos compostos fenólicos, não só devido à temperatura elevada, mas também devido ao corte das células dos tecidos, que facilita as enzimas de acederem a este tipo de compostos (Cheynier, 2005).

Nas ameixas secas, o conteúdo em flavonóis e ácidos hidroxicinâmicos diminui para praticamente metade em relação às ameixas não processadas, enquanto que os flavanóis não são detectados nos produtos processados. Esta redução no conteúdo em compostos fenólicos é indicativa da sua extensa degradação com o processamento (Donovan *et al.*, 1998; Piga *et al.*, 2003). O processamento térmico promove a degradação dos ácidos hidroxicinâmicos, o que está directamente relacionado com o aumento da actividade da enzima polifenol oxidase. Esta degradação é maior para temperaturas de secagem inferiores, pois estas são mais próximas da temperatura óptima da actividade da enzima. Para temperaturas mais elevadas, superiores a 70 ºC, ocorre inactivação completa da polifenol oxidase. No entanto, o inverso acontece com a degradação dos flavonóides, em que a sua diminuição é proporcional ao aumento de temperatura, indicando que a degradação destes compostos não está relacionada com a acção da polifenol oxidase (Piga *et al.*, 2003).

3. FUNDAMENTOS SOBRE MICROEXTRACÇÃO EM FASE SÓLIDA E TECNOLOGIA DE ALTA PRESSÃO

3.1. Microextracção em fase sólida (SPME)

A microextracção em fase sólida (SPME) é uma técnica relativamente nova, da década de 90, para a extracção e concentração simultânea de analitos presentes numa amostra. As principais vantagens da SPME são a elevada sensibilidade, simplicidade de utilização, rapidez e a não utilização de solventes orgânicos. Todas estas vantagens fazem com que a SPME seja amplamente utilizada em diversas áreas como análise ambiental, solos, água, alimentos, produtos naturais e farmacêuticos e análise clínica (Kataoka *et al.*, 2000; Mester *et al.*, 2001). Esta técnica continua em permanente evolução em relação ao processo de extracção e às aplicações.

A metodologia de SPME consiste na extracção e concentração de um analito directamente da amostra usando uma fase estacionária apropriada. Esta fase estacionária pode estar a revestir diversos tipos de suportes, como as fibras de sílica fundida, as paredes de um pequeno tubo, as paredes de um recipiente de amostragem, a superfície de partículas, um agitador magnético e discos ou membranas (Pawliszyn, 2000). O tipo de suporte usado varia consoante a amostra e o tipo de analito a analisar, assim como o objectivo da análise.

A fibra de sílica fundida é o dispositivo mais usado pois é muito versátil, podendo ser usado em amostras gasosas, líquidas e sólidas, e também se pode facilmente adaptar o equipamento de análise e quantificação dos compostos extraídos. Este dispositivo consiste num suporte, idêntico a uma seringa, em que no interior da agulha está localizada a fibra de sílica fundida (Figura 18). A fibra está revestida por um filme de fase estacionária com uma espessura muito reduzida (máximo 100 μm). Estas dimensões do revestimento da fibra extraem pequenas quantidades de compostos permitindo uma correcta identificação e/ou quantificação e compostos em reduzidas quantidades de amostra (Pawliszyn, 2000).

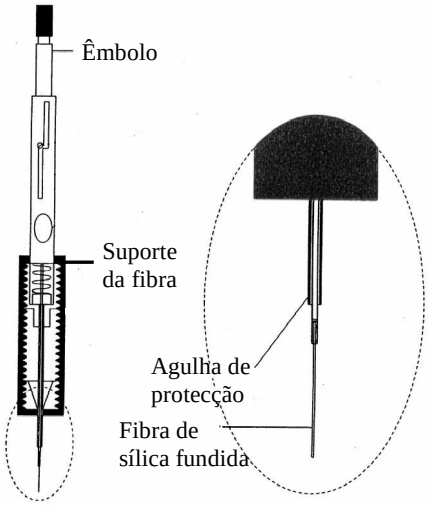


Figura 18 – Dispositivo de SPME de fibra de sílica fundida (adaptado de Kataoka *et al.*, 2000).

Normalmente o procedimento utilizado para a análise de uma amostra usando a fibra de SPME consiste em colocar a amostra num recipiente fechado com uma tampa contendo um septo, por onde a agulha do dispositivo de SPME perfura. A fibra revestida é exposta à amostra e a extracção dos compostos pode ser realizada de duas formas, por imersão do revestimento da fibra numa amostra líquida ou por contacto com a fase de vapor de amostras sólidas, líquidas ou gasosas. Depois da exposição da fibra durante o tempo necessário para a extracção, esta é retirada do contacto com a amostra e os compostos são desorvidos para posterior separação, identificação e/ou quantificação (Kataoka *et al.*, 2000). A Figura 19 esquematiza um exemplo de um procedimento de extracção por contacto da fibra de SPME com a fase de vapor da amostra e desorção térmica dos compostos extraídos no injector de um cromatógrafo. Esta metodologia de análise da fase de vapor possui a vantagem de permitir, por exemplo, a análise dos compostos voláteis e semi-voláteis dos alimentos que são perceptíveis pelo sistema olfactivo.

A técnica de SPME é um processo baseado em equilíbrios simultâneos em sistemas multifásicos. A transferência dos compostos da amostra para a fase estacionária inicia logo que a fibra é exposta directamente na amostra ou no espaço de cabeça. A extracção dos compostos termina quando a concentração dos compostos atinge um equilíbrio de distribuição entre a amostra e o revestimento da fibra, o que significa que uma vez atingido o equilíbrio a quantidade de compostos extraídos é constante e independente do aumento do tempo de extracção. Em teoria, a transferência de massa num sistema trifásico

(fibra/espaço de cabeça/amostra) depende dos equilíbrios de partição entre as três fases, dos coeficientes de difusão e das dimensões das fases. No entanto, os sistemas reais são mais complexos pois os compostos podem interagir entre si, com as paredes do frasco e eventualmente com a sílica fundida da fibra (Mester *et al.*, 2001).

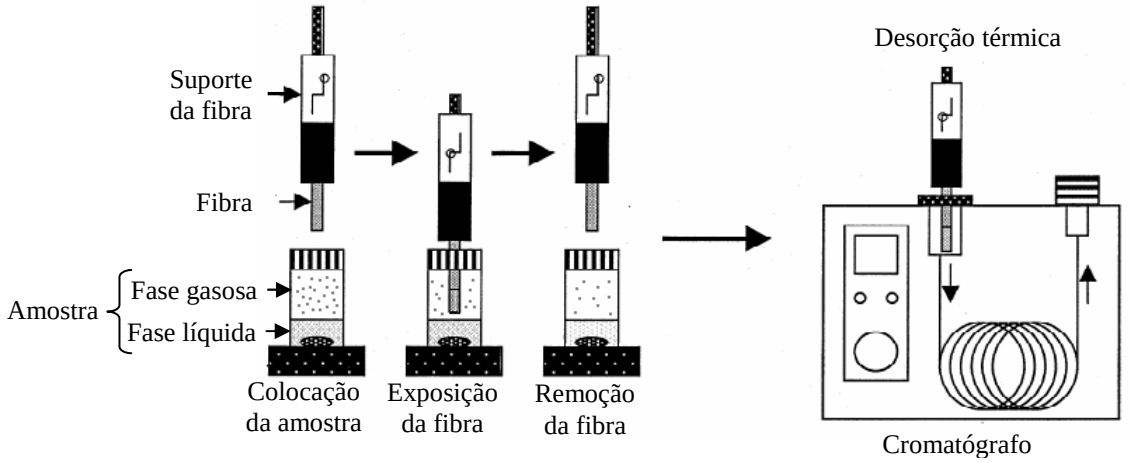


Figura 19 – Procedimento de extracção por exposição da fibra de SPME à fase de vapor da amostra e desorção térmica dos compostos (adaptado de Kataoka *et al.*, 2000).

A selecção do revestimento da fibra de SPME é um procedimento muito importante, pois cada um possui uma eficiência de extracção distinta para compostos de diferentes características físico-químicas, apresentando também limites de detecção e gamas de linearidade distintas (Kataoka et al., 2000). O revestimento da fibra de SPME pode ser líquido ou sólido, possuindo diferentes equilíbrios de extracção, por absorção ou adsorção. Independentemente da natureza da fase estacionária, o processo de extracção começa pela adsorção dos analitos à superfície da fase estacionária e, em seguida, os analitos difundem para o seu interior. Se os coeficientes de difusão forem reduzidos, os analitos permanecerão mais tempo na superfície da fase estacionária, ocorrendo uma extracção por adsorção. Se os coeficientes de difusão dos analitos forem elevados, existe a partição total entre a fase estacionária e a matriz, ocorrendo uma extracção por absorção (Pawliszyn, 2000).

Os revestimentos do tipo adsorvente são formados por sólidos porosos ou com elevada área superficial, onde a extracção é efectuada através da sorção dos analitos aos poros internos, de menores dimensões (microporos, 2-20 Å e mesoporos, 20-500 Å), ou

externos, de maiores dimensões (macroporos > 500 Å) e que se encontram principalmente na superfície (Figura 20). A vantagem dos revestimentos adsorventes está relacionada com a maior selectividade, maior limite de detecção e capacidade para a retenção de compostos polares voláteis. No entanto, apresenta algumas limitações quando a concentração dos analitos é elevada, pois pode ocorrer a saturação da superfície disponível para adsorção, o que poderá originar relações não lineares entre a concentração dos analitos na matriz e a quantidade de analitos extraída (Pawliszyn, 2000). Os revestimentos absorventes são formados por polímeros líquidos em que, teoricamente, os analitos podem entrar e sair livremente da fase estacionária (Figura 20). Esta propriedade é uma vantagem importante em relação aos revestimentos do tipo adsorvente, pois apresentam linearidade entre a concentração dos analitos na matriz e a quantidade de analitos extraída para uma gama de concentrações de analitos e interferentes superior (Pawliszyn, 2000).

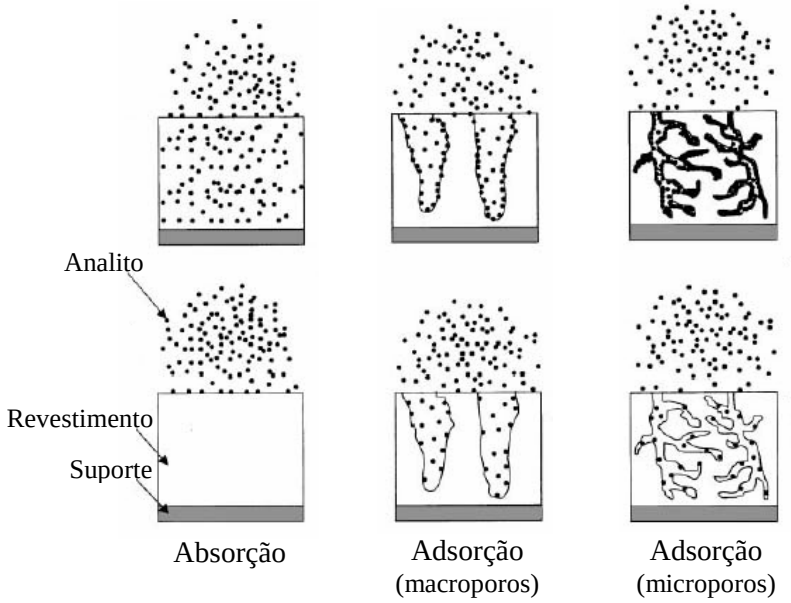


Figura 20 – Esquema da extracção por absorção e adsorção das fases estacionárias da fibra de SPME (adaptado de Pawliszyn *et al.*, 2000).

Diversos tipos de revestimentos de fibras de SPME estão disponíveis comercialmente com diferentes polaridades, modos de extracção e espessuras de filme. O tipo de fibra de SPME deve ser seleccionado consoante o tipo de compostos que se pretende analisar. A afinidade de uma fibra para um composto depende da polaridade. Normalmente, fibras

com revestimentos polares têm maior afinidade para compostos polares e vice-versa. O revestimento das fibras pode ser um líquido, um sólido cristalino (que se torna líquido com o aumento da temperatura) ou um líquido com partículas porosas. Neste último tipo de revestimento existe um aumento na capacidade de retenção dos compostos devido ao efeito combinado de adsorção e distribuição pela fase estacionária (Kataoka *et al.*, 2000). As espessuras do revestimento da fibra de SPME podem variar de 7 a 100 μm, em que os revestimentos mais finos são mais apropriados para compostos semi-voláteis e os mais espessos para compostos voláteis. As fibras de SPME com revestimentos mais espessos requerem mais tempo para atingir o equilíbrio, mas proporcionam maior sensibilidade devido à grande quantidade de analitos que podem ser extraídos (Kataoka *et al.*, 2000; Mester *et al.*, 2001).

A quantidade de compostos extraídos pela fibra de SPME é também bastante influenciada pelas condições experimentais e pela matriz líquida onde se encontram os compostos. A extracção depende de factores como o tempo, a temperatura de extracção, a agitação, a adição de sal, o volume e a composição da amostra (Kataoka et al., 2000; Mester et al., 2001). O tempo de extracção é principalmente determinado pelo coeficiente de partição do analito entre o revestimento da fibra e a amostra. A temperatura de extracção e a agitação da amostra são importantes para promover a passagem dos compostos voláteis para o espaço de cabeça, diminuindo o tempo necessário para a sua extracção. A adição de sais à amostra melhora a eficiência de extracção devido ao efeito de "salting-out", pois a saturação da amostra promove também a passagem dos compostos para o espaço de cabeça. A razão entre o volume da amostra e o volume da fase de vapor é também determinante para a quantidade de compostos extraídos, pois influencia os equilíbrios de partição entre as fases. A composição da amostra influencia a extracção devido à interacção que pode existir entre os diferentes compostos que constituem a matriz (Kataoka *et al.*, 2000).

A desorção dos compostos extraídos pela fibra de SPME é também um passo importante do procedimento. Uma eficiente desorção térmica depende da volatilidade dos compostos, da espessura do revestimento da fibra, da temperatura e do tempo de exposição da fibra de SPME.

3.2. Tecnologia de alta pressão no processamento de alimentos

O objectivo principal do processamento de alimentos é a sua preservação durante um longo período de tempo. Na maioria dos métodos tradicionais de preservação, os alimentos são submetidos a altas temperaturas por um certo período de tempo para a inactivação de enzimas e microorganismos patogénicos. O aquecimento provoca alterações indesejáveis nos produtos tais como a redução das propriedades nutritivas e modificações de textura, cor, aroma e sabor. Actualmente, a tendência do consumo de alimentos é na direcção de produtos minimamente processados, livres de aditivos, mais seguros, estáveis no armazenamento e com melhor qualidade em termos de textura, cor, aroma e sabor. Estes requisitos sugerem a utilização na indústria alimentar de tratamentos não térmicos como complementares ou alternativos aos tradicionais tratamentos térmicos.

O processamento por alta pressão é uma tecnologia inovadora no processamento de alimentos sensíveis aos tratamentos térmicos, como os frutos e os vegetais. Esta tecnologia consiste em submeter o alimento a altas pressões com o objectivo de destruir microrganismos e inactivar enzimas para a sua preservação durante longos períodos de tempo, mas mantendo algumas das propriedades organolépticas e nutricionais do alimento não processado.

O processamento de alimentos por alta pressão é referido como uma tecnologia inovadora. Contudo, tratamentos com alta pressão foram reconhecidos como uma técnica potencial de preservação há cerca de um século, nomeadamente no processamento de alguns frutos (Hite *et al.*, 1914). Apesar deste conhecimento sobre o efeito da alta pressão na inactivação dos microorganismos, o uso da alta pressão na preservação de alimentos só começou a ser utilizado a nível industrial e comercial nos últimos 20 anos, primeiro no Japão e mais tarde na Europa. Uma grande variedade de produtos tratados por alta pressão está actualmente disponível, maioritariamente no mercado japonês e americano, como por exemplo sumos de frutos, molhos e produtos cárneos. Na Europa esta tecnologia é ainda pouco utilizada pela indústria, sendo principalmente usada para a preservação de sumos de frutos.

No processamento por alta pressão, alimentos líquidos ou sólidos, são submetidos a pressões entre 100 e 800 MPa. O alimento pode estar acondicionado em embalagem flexível, sendo inserido num fluído de baixa compressibilidade (geralmente água), que funciona como meio de transferência da pressão. A pressurização é realizada num espaço confinado, vaso de compressão, e o nível de pressão é mantido ao longo da duração do processo, que pode variar dos milissegundos aos 20 minutos. O processamento de produtos líquidos pode ser realizado através de um sistema semicontínuo, utilizando três vasos de compressão e um sistema de válvulas automáticas, em que no fim o produto é embalado em condições assépticas (Ting e Marshall, 2002). O equipamento de alta pressão tem um custo elevado, sendo esta a principal razão pela qual a sua aplicação é ainda limitada no processamento de alimentos. Contudo, o desenvolvimento tecnológico deste tipo de equipamento tende a tornar os custos do processamento mais competitivos em relação aos métodos de conservação tradicionais.

A alta pressão baseia-se em dois princípios gerais, o de Le Chatelier e o isostático. O princípio de Le Chatelier diz que qualquer fenómeno (transição de fase, mudança de conformação molecular ou reacção química) acompanhado por uma redução de volume é favorecido pelo aumento de pressão. O segundo princípio, o isostático, descreve que a pressão é transmitida de uma forma uniforme e quase instantânea através do alimento, sendo independente do seu tamanho e geometria (Cheftel, 1995; Knorr *et al.*, 1998). Esta é uma das principais vantagens do processamento por alta pressão em relação ao tratamento térmico, em que a distribuição da temperatura não é uniforme sendo necessário aumentar o tratamento para que seja atingida a temperatura desejada no interior do alimento, o que provoca a perda de qualidade do produto final (San Martín *et al.*, 2002).

O aumento da pressão durante o processamento provoca o aumento uniforme da temperatura dos alimentos devido ao aquecimento adiabático. A amplitude deste aumento de temperatura depende da temperatura inicial do produto e da sua composição. A água aumenta cerca de 3 ºC por um aumento de 100 MPa, enquanto que se o alimento contiver gordura a temperatura pode aumentar até 6 ºC por 100 MPa (Ting *et al.*, 2002; Balasubramanian e Balasubramaniam, 2003). O tratamento com alta pressão pode também ser realizado com temperatura elevada (até cerca de 100 ºC) originando os mesmos efeitos em termos de segurança que os tratamentos térmicos convencionais (pasteurização e

esterilização) mas com menores consumos de energia e com uma melhoria da qualidade do produto final.

Outro método de preservação dos alimentos é a conservação a temperaturas reduzidas (inferiores a 0 ºC), em que o crescimento de microorganismos e muitas das reacções químicas e enzimáticas são inibidos. Os alimentos podem também ser congelados sob pressão, sendo um processo de congelação ultra rápido devido à despressurização instantânea a baixas temperaturas em que a principal vantagem é a formação muito rápida de cristais de gelo com dimensões muito reduzidas sem o aumento de volume do gelo durante a transição de fases (Otero *et al.*, 2002). Por outro lado, a influência da pressão nas propriedades físicas da água faz com que a temperaturas inferiores a 0 ^{o}C e a pressões moderadas (a cerca de 200 MPa), a água permaneça em estado líquido (Otero *et al.*, 2002). Esta propriedade da água a altas pressões, assim como as características do gelo formado, originam uma menor degradação da estrutura dos tecidos e consequentemente da textura dos frutos e vegetais em comparação com as técnicas de congelação tradicionais.

A pressão aplicada, o tempo de aplicação e a temperatura do processamento dependem do tipo do produto a ser tratado e do produto final desejado. Os principais efeitos da pressão nos alimentos são a inactivação dos microorganismos e a modificação das estruturas tridimensionais dos polímeros (proteínas e polissacarídeos). A redução do volume com o aumento da pressão ocorre devido à formação ou ruptura de ligações não covalentes (pontes de hidrogénio, ligações iónicas e ligações hidrofóbicas) e dos rearranjos com as moléculas de solvente. As propriedades organolépticas (cor, textura, aroma e sabor) e nutricionais praticamente não se alteram, pois o processamento por alta pressão não afecta as ligações químicas covalentes (Smelt, 1998).

3.2.1. Efeitos do processamento por alta pressão

A capacidade do processo de alta pressão de destruir e/ou inactivar microorganismos varia de acordo com o nível de pressão e tempo de tratamento, o produto a ser processado e com o tipo de microorganismo contaminante. Os fungos, leveduras e células vegetativas são, em geral, sensíveis ao tratamento por alta pressão entre 200 e 600 MPa, sendo que taxa de destruição dos microorganismos aumenta com o aumento da pressão ou do tempo

de processamento. No entanto, certos esporos bacterianos apresentam elevada resistência ao tratamento a pressões até 1000 MPa (Cheftel, 1995; Smelt, 1998). Nesse caso, a combinação do tratamento a alta pressão com o tratamento térmico a temperatura moderada, cerca de 60^{o}C, podem promover uma completa inactivação microbiana. Outra alternativa de processo consiste em induzir a germinação de esporos a baixa pressão (200 MPa), com a posterior destruição das células vegetativas germinadas a alta pressão que se tornaram menos resistentes à pressão (Cheftel, 1995). Os alimentos com pH baixo, como é o caso dos frutos, não possuem este problema pois a germinação de esporos bacterianos, resistentes à pressão, é inibida nestas condições (Farr, 1990).

A inactivação microbiana com o aumento da pressão parece estar principalmente relacionada com as alterações na membrana citoplasmática. As membranas são compostas por uma camada de fosfolípidos envolvidos por proteínas funcionais que, entre outras funções, têm um papel importante no transporte de iões e outros nutrientes para as células. A pressão provoca a alteração estrutural da camada de fosfolípidos e algumas desnaturações proteicas, modificando a permeabilidade e a selectividade da membrana citoplasmática, podendo resultar na morte da célula (San Martín *et al.*, 2002). Outras mudanças morfológicas foram observadas na célula, tais como a compressão de vacúolos, alongamento da célula, separação da membrana da parede celular, contracção da parede celular com a formação de poros, modificações no núcleo e em organelos intracelulares, coagulação de proteínas citoplasmáticas, libertação de constituintes intracelulares para o exterior da célula, entre outros (Cheftel, 1995).

No caso das enzimas, geralmente pressões acima de 300 MPa à temperatura ambiente são suficientes para ocorrer uma inactivação irreversível na maioria das enzimas. A pressões inferiores a 200 MPa, certas enzimas são inactivadas enquanto que outras podem ser activadas, pois a desnaturação das enzimas pode ser reversível. As enzimas são proteínas em que a actividade biológica é devida a um local activo mantido pela conformação tridimensional da molécula. Pequenas mudanças no local activo podem levar a uma perda ou aumento da actividade da enzima ou mudanças na especificidade do substrato. As proteínas são estruturas delicadas, mantidas por interacções entre a cadeia proteica e pelas interacções com o solvente. Assim, mudanças nos factores externos, como pressão e temperatura, podem perturbar o complexo balanço das interacções intramoleculares e, consequentemente, podem levar ao desdobramento e/ou desnaturação

da cadeia polipeptídica. O aumento da pressão provoca mudanças conformacionais na estrutura das enzimas e consequentemente na sua funcionalidade (Hendrickx *et al.*, 1998).

Os tratamentos com pressões até 350 MPa, no geral, não afectam a textura e a estrutura dos tecidos dos frutos e vegetais. A pressões baixas (100 MPa) pode ocorrer uma perda instantânea da textura devido à compressão sem ruptura da estrutura celular, enquanto que para pressões mais elevadas (> 200 MPa) as perdas de textura podem ser gradualmente recuperadas e alguns produtos podem ficar mais firmes. Este efeito pode ser explicado devido a acção da enzima pectina metilesterase que pode ser só parcialmente inactivada com os tratamentos por pressão. Os polissacarídeos pécticos desesterificados pela enzima podem associar-se com os iões cálcio, o que aumenta a firmeza dos tecidos (Basak e Ramaswamy, 1998). O processamento por alta pressão pode também induzir a formação de géis em alguns produtos alimentares como resultado da desnaturação de proteínas ou gelatinização de amido.

Na maioria dos alimentos processados por alta pressão a cor, o aroma e o sabor são mantidos, pois a maioria das enzimas são inactivadas. Por outro lado, as reacções de Maillard e a formação dos compostos responsáveis pelo aroma a cozido e pela alteração da cor também não ocorrem durante o tratamento por pressão. Mesmo em tratamentos por alta pressão com temperaturas elevadas, o aroma e sabor do produto final é menos alterado em comparação com os tratamentos térmicos convencionais (Ting *et al.*, 2002).

O conteúdo em vitaminas e em compostos fenólicos, com propriedades biológicas benéficas, praticamente não é alterado para tratamentos com pressões que variam entre 100 e 600 MPa e para temperaturas até os 65 ºC. A preservação destes compostos pode dever-se ao facto de que a alta pressão inactiva as enzimas responsáveis pela degradação dos compostos fenólicos e também não afecta as ligações covalentes (Butz *et al.*, 2003).

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CAPÍTULO III

1. AMOSTRAS

Com vista a cumprir os objectivos delineados para este trabalho, analisaram-se amostras de ameixas da variedade 'Rainha Cláudia Verde' em fresco e ao longo do processo de confitagem. As ameixas foram cedidas pela organização de produtores de ameixa na região do Alto Alentejo, a Fruteco - Fruticultura Integrada Lda., responsável pela Denominação de Origem Protegida Ameixa d'Elvas. As ameixas foram confitadas na Confibor – Transformação Agro-alimentar Lda., em Estremoz.

Os pomares que produzem a Ameixa d'Elvas usada neste trabalho possuem ameixeiras enxertadas nos porta-enxertos GF 8.1 (espécie *Prunus mariana*) com o clone PUE 11 da variedade 'Rainha Cláudia Verde'. O clone foi seleccionado na Universidade de Évora e caracteriza-se por apresentar um bom vigor, com uma ramificação média e uma boa produção. Os pomares foram plantados com uma distância entre árvores de 6 x 4 m, sendo a copa das árvores conduzida em forma de vaso. As ameixeiras são auto-inférteis, isto é, não se auto-polinizam, necessitando de outras árvores polinizadoras. As ameixeiras polinizadoras foram plantadas numa proporção de 10%, sendo a variedade seleccionada a 'Stanley' pois teve uma boa adaptação ao clima da região e a floração era simultânea e compatível com a da 'Rainha Cláudia Verde'. Os pomares possuem rega automática, de gota a gota, com um fluxo de 3 L/h em cada árvore. Normalmente, não são adicionados fertilizantes aos solos e as ameixeiras não são tratadas com produtos fitofarmacêuticos.

Com o objectivo de estudar as alterações físico-químicas que ocorrem com a confitagem e que se possam relacionar com as diferenças na qualidade da Ameixa d'Elvas confitada, foram seleccionados dois pomares de fenótipo semelhante, Vila Viçosa e Cano. Os pomares foram seleccionados devido ao conhecimento prévio do comportamento díspar das ameixas com a confitagem. O pomar de Vila Viçosa tem um solo de origem xistosa com uma textura ligeira e elevados teores em argila, enquanto que no pomar do Cano o solo é de origem calcária, com uma textura pesada e estrutura granulosa. A capacidade de troca catiónica é baixa nos solos de Vila Viçosa e mediana a elevada no Cano, o que significa que o solo do Cano consegue mais facilmente conduzir os iões para a árvore. As

análises químicas ao solo, no ano de plantação dos dois pomares, revelaram a existência de níveis similares de fósforo e magnésio. No entanto, foram determinados no pomar de Vila Viçosa em relação ao Cano níveis inferiores de cálcio, potássio e nitratos. O pH do solo em ambos os pomares era de cerca de 7,5. O solo do pomar do Cano é mais rico do que o de Vila Viçosa, originando ameixeiras com um maior vigor do que em Vila Viçosa.

No ano de 2003, as ameixas foram colhidas nos dois pomares no estado de maturação adequado para a confitagem, avaliado pelos parâmetros convencionais: aspecto visual (cor verde), sólidos solúveis totais (16 – 17 ºBrix), acidez total (pH 3.3) e acidez titulável (1% de ácido málico). As ameixas do pomar de Vila Viçosa foram colhidas 7 dias antes das do Cano, pois neste pomar a maturação dos frutos estava atrasada segundo estes parâmetros. As ameixas foram confitadas na empresa, sendo todo o processo seguido para não ocorrerem diferenças significativas durante o processamento entre as ameixas provenientes dos dois pomares. A recolha das amostras foi realizada em duas etapas do processamento, após o cozimento do fruto e no fim do processamento (produto final). Os frutos do pomar de Vila Viçosa deram origem a produtos de boa qualidade (Figura 21a), enquanto que os frutos do pomar do Cano originaram produtos com menor firmeza, de qualidade inferior e sem valor comercial (Figura 21b). Nas ameixas não processadas, cozidas e confitadas dos dois pomares foram estudadas a microestrutura dos tecidos, a textura dos frutos e a composição em polissacarídeos da parede celular. A actividade enzimática das enzimas poligalacturonase, pectina metilesterase e celulase foi determinada nas ameixas não processadas dos dois pomares.

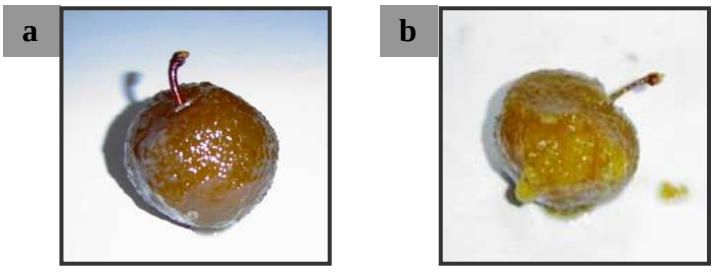

Figura 21 – Amostras representativas de ameixas confitadas, em 2003, dos pomares de (a) Vila Viçosa e (b) Cano.

Para confirmar as conclusões obtidas pelas análises físico-químicas, voltaram a ser colhidas ameixas em 2005 nos dois pomares, Vila Viçosa e Cano, em dois estados de maturação diferentes. Os dias de colheita foram seleccionados de acordo com o estado de maturação ideal para confitar as ameixas do pomar de Vila Viçosa (19 de Julho) e o estado de maturação ideal para processar as ameixas do pomar do Cano (26 de Julho). A diferença entre as duas colheitas foi de 7 dias, pois nesse ano a maturação das ameixas do Cano estava também atrasada em relação às ameixas de Vila Viçosa. Os estados de maturação foram determinados pelos parâmetros convencionais (sólidos solúveis totais, acidez total e acidez titulável).

As ameixas obtidas em fresco nos dois dias de colheita em 2005, de ambos os pomares, foram confitadas no laboratório seguindo o processamento utilizado na empresa. As amostras foram recolhidas nas mesmas etapas do processamento, após o cozimento e confitadas. O aspecto visual revelou que as ameixas recolhidas nos mesmos dias, em ambos os pomares, tinham uma aparência semelhante depois do processo de confitagem. As ameixas colhidas no dia 19 de Julho apresentavam uma boa qualidade visual (Figura 22), enquanto que as ameixas colhidas no dia 26 de Julho apresentavam, após o processamento, fracturas na epiderme e com uma aparente má qualidade (Figura 23). Nas ameixas não processadas, cozidas e confitadas, dos dois estados de maturação e dos dois pomares foram realizadas análises de microestrutura e textura.

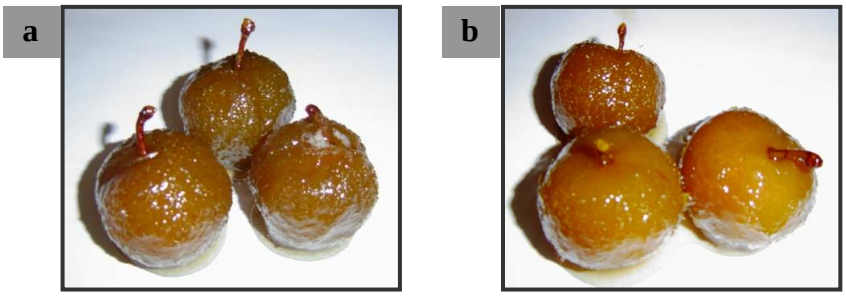

Figura 22 – Amostras representativas de ameixas confitadas obtidas a partir de frutos colhidos em 19 de Julho de 2005 nos pomares de (a) Vila Viçosa e do (b) Cano.

No ano de 2005, no pomar de Vila Viçosa foram também colhidas ameixas em mais dois estados de maturação, 6 dias antes (13 de Julho) e 4 dias depois (22 de Julho) do primeiro dia de colheita dos frutos para confitar. Em todas as ameixas não processadas foi

quantificada a actividade das enzimas poligalacturonase, pectina metilesterase e celulase, com o objectivo de estudar a evolução da actividade destas enzimas ao longo da maturação das ameixas.

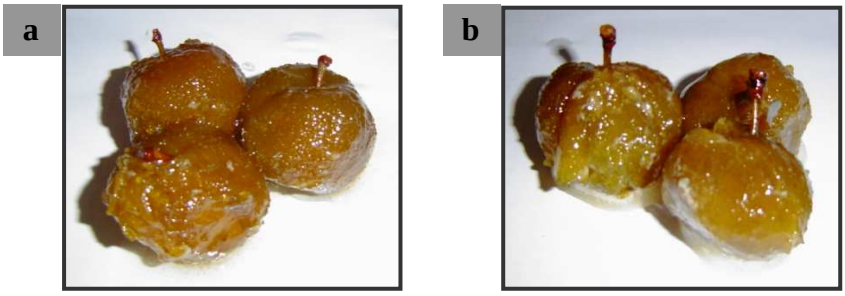

Figura 23 – Amostras representativas de ameixas confitadas obtidas a partir de frutos colhidos em 26 de Julho de 2005 nos pomares de (a) Vila Viçosa e do (b) Cano.

O método proposto para avaliar de uma forma fácil e rápida a altura ideal para a colheita das ameixas para serem processadas foi testado com Ameixas d'Elvas não processadas colhidas em dois anos. Foram utilizadas ameixas do ano de 2003 em 5 estados de maturação diferentes para ambos os pomares, Vila Viçosa e Cano. Os estados de maturação foram determinados pelos parâmetros convencionais (sólidos solúveis totais, acidez total e acidez titulável). As ameixas não processadas de 2005 foram também usadas para testar o método proposto para seleccionar as ameixas para confitar.

A enzima pectina metilesterase foi purificada, caracterizada bioquimicamente e a sua estabilidade térmica e a tratamentos de alta pressão foi também avaliada. Neste estudo foram usadas ameixas colhidas em 2004 no pomar de Vila Viçosa, num estado de maturação óptimo para serem processadas (16 ºBrix, pH 3.1 e 1% de ácido málico).

A determinação da composição em compostos fenólicos da ameixa não processada foi realizada em amostras de ambos os pomares (Vila Viçosa e Cano) colhidas em 2006 com similar estado de maturação, avaliado pelos parâmetros convencionais: 16 a 17 ºBrix, pH 3.3 e 1% de ácido málico.

No estudo da composição volátil das ameixas confitadas e da calda de açúcar foram usadas amostras cedidas pela empresa Confibor no ano de 2002. As ameixas confitadas permaneceram à temperatura ambiente na calda de açúcar onde tinham sido processadas até serem analisadas.

SIMPLE AND SOLVENT-FREE METHODOLOGY FOR SIMULTANEOUS QUANTIFICATION OF METHANOL AND ACETIC ACID CONTENT OF PLANT POLYSACCHARIDES BASED ON HEADSPACE SOLID PHASE MICROEXTRACTION-GAS CHROMATOGRAPHY (HS-SPME-GC-FID)

CAPÍTULO IV

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Simple and solvent-free methodology for simultaneous quantification of methanol and acetic acid content of plant polysaccharides based on headspace solid phase microextraction-gas chromatography (HS-SPME-GC-FID)

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Abstract

A simple and solvent-free methodology is proposed for the simultaneous determination of methanol and acetic acid content of any type of plant cell wall polysaccharides. The methanol and acetic acid, released by saponification, are extracted from the headspace of the aqueous solution by solid phase microextraction (HS-SPME) using a DVB/Carboxen/PDMS fibre. The analytes are separated by gas chromatography and detected using a flame ionization detector (GC-FID). The quantification of methanol and acetic acid is done using external calibration curves. A linear relationship between the concentration of methanol (40–100 mg L^{-1}) and acetic acid (25–105 mg L^{-1}) and their GC peak area was observed (r^2Z0.987 and 0.988, respectively) with a reproducibility of 10%. HS-SPME-GC-FID revealed to be a clean, simple, fast and reliable methodology for the determination of the methanol and acetic acid content of cell wall polysaccharide extracts.

Keywords: Methanol; Acetic acid; Solid phase microextraction; Headspace analysis; Plant polysaccharides

1. Introduction

Acetylation of plant cell wall polysaccharides decreases the interpolymer binding, leading to weak and unstable cell walls (Aspinall, 1980; Kunzek, Kabbert, & Gloyna, 1999; Pilnik & Rombouts, 1985; Van Buren, 1979; Wakabayashi, 2000). In pectic polysaccharides, the number and distribution of methyl and acetyl groups of galacturonic acid residues influence widely their ion-exchange properties, water-binding capacity, cross-linking through calcium ions and hydrogen bonding. Consequently, these substitutions are key parameters for understanding and predicting the role of these polysaccharides within the cell walls, namely, their contribution to the texture of fruits during ripening, storage and processing (Voragen, Pilnik, Thibault, Axelos, & Renard, 1995).

Several methods have been developed to quantify the methyl groups in pectic polysaccharides. The Food Chemical Codex (1981) proposes a titrimetric method for determination of the degree of methylesterification (DM) of pectins. This procedure involves titration with sodium hydroxide of a polysaccharide suspension before and after alkali hydrolysis. Because acetyl groups are usually present, the DM value can be only obtained after correction for the acetyl groups by a separated specific determination. Another possible disadvantage of this method is the need of relatively large quantities of sample (ca. 30 mg). The amount of methanol can also be quantified by colorimetry (Wood & Siddiqui, 1971), alcohol oxidase enzymatic assay (Klavons & Bennett, 1986) and capillary zone electrophoresis (Jiang, Liu, Wu, Chang, & Chang, 2005). Direct determination of DM using ^{1}H NMR (Grasdalen, Bakøy, & Larsen, 1988; Renard & Jarvis, 1999) and FT-IR (Barros et al., 2002; Chatjigakis et al., 1998; Gananasambandam & Proctor, 2000; Monsoor, Kalapathy, & Proctor, 2001) is also a possibility. However, for the simultaneous determination of methanol and acetic acid, gas chromatography (Huisman, Oosterveld, & Schols, 2004; McFeeters & Armstrong, 1984; Waldron & Selvendran, 1990) and HPLC (Levigne, Thomas, Ralet, Quemener, & Thibault, 2002; Voragen, Schols, & Pilnik, 1986) are the most frequently used methodologies.

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Solid-phase microextraction (SPME) is a sample preparation technique that is simple to use, relatively fast and does not require solvent extraction (Arthur & Pawliszyn, 1990; Zhang & Pawliszyn, 1993). It has been successfully applied to a wide variety of compounds, in particular for the extraction of volatile and semi-volatile compounds from environmental, biological and food samples, since SPME is available with various sorbent materials and various coating thickness (Kataoka, Lord, & Pawliszyn, 2000; Mester, Sturgeon, & Pawliszyn, 2001). The use of solid phase microextraction (SPME) coupled with gas chromatography–mass spectrometry (GC–MS) was also proposed for the simultaneous determi-nation of the degrees of methylation and acetylation in 1 mg of fruit and vegetable pectic polysaccharides, using a Carboxen/PDMS SPME fibre (Savary & Nuñez, 2003). In this study, deuterated isotopomers were used as internal standards and for construction of calibration curves were used concentrations between 0.1 and 8.0 mmol mL^{-1} (3–260 mg L^{-1}) for methanol and 0.05 and 2.0 mmol mL^{-1} (3–120 mg L^{-1}) for acetic acid.

In order to propose a simple methodology and to avoid the use of a mass detector and deuterated species, in this work it is proposed a new methodology for the simultaneous quantification of methanol and acetic acid released from polysaccharides after saponification. The headspace of the samples were analysed by SPME using a GC equipped with a flame ionisation detector (FID). Different coating fibres (DVB/Carboxen/PDMS and PDMS) and times of extraction (15, 30 and 45 min) were tested. For comparative purposes and validation of the SPME methodology, methanol and acetic acid contents in polysaccharide samples were also determined by direct injection of the aqueous solution in the GC-FID.

2. Materials and methods

2.1. Materials

Standards used were of analytical grade. Methanol (99.0%) and acetic acid (99.5%) were purchased from Fluka and Riedel, respectively. Calibration curves were made from readily prepared methanol (0.8 g L^{-1}) and acetic acid (1 g L^{-1}) stock solutions. A SPME holder from Supelco, Inc. (Bellefonte, PA, USA) was used to perform headspace-SPME manually. SPME fibres (Supelco, Inc., Belfont, PA, USA) coated with 50/30 μm divinylbenzene/ Carboxen on polydimethylsiloxane (DVB/Carboxen/ PDMS) and 100 μm polydimethylsiloxane (PDMS) were conditioned prior to use at 250 ºC for 4 and 0.5 h, respectively, according to the manufacturer's recommendations.

2.2. Sample origin and preparation

Polysaccharides were obtained from pulp washing water and from alcohol insoluble residue (AIR) of plums (*Prunus domestica*, var. 'Rainha Claudia'). Pectic polysaccharide-rich samples were also obtained by extraction of AIR with water and 0.5 M imidazole/HCl pH 7.0, as described by Mafra et al. (2001). Each sample, 2 mg for water and imidazole extracts and 5 mg for AIR, was dispersed in water (2.4 mL) in vials with 10 mL capacity and sonicated for 10 min in a water bath at room temperature. The saponification of polysaccharides occurred by the addition of 0.8 mL of 2 M NaOH, with a reaction time of 1 h at 25 ºC, as described by Waldron and Selvendran (1990). The reaction was finished by the addition of 0.8 mL of 2 M HCl, and the pH was adjusted to 2.0.

2.3. Determination of methanol and acetic acid by HS-SPME-GC-FID

The vials (10 mL) containing 4.0 mL of sample suspension (sample dispersed in 2.4 mL of water, 0.8 mL of 2 M NaOH and 0.8 mL of 2 M HCl) or standard solutions were thermostatized at 40 ºC in a water bath, with continuous stirring. After 15 min, the SPME fibre was manually inserted through the Teflon septum into the headspace of the vial. Since headspace volume can be a critical factor determining the precision of the results in three-phase systems, vials from the same producer and lot were used.

For HS-SPME optimization, for both DVB/Carboxen/PDMS and PDMS fibre coatings, three times of extraction were used, 15, 30 and 45 min. The analysis of the samples was performed using the DVB/Carboxen/PDMS fibre coating exposed at 40 ºC during 30 min.

The SPME coating fibre containing the headspace volatile compounds was introduced into the GC injection port at 250 ºC and kept for 10 min for the desorption. A Hewlett-Packard 5890 series II gas chromatograph (Hewlett-Packard, Wilmington, USA), equipped with a split/splitless injector and a flame ionisation detector (FID) was used. The injection port was lined with a 0.75 mm i.d. splitless glass liner. Splitless injection mode was used (3 min). The desorbed compounds were separated in a 30 m length DB-Wax column (J&W) with 0.53 mm i.d. and 1.0 μm film thickness and hydrogen as carrier gas was used at 6 mL/min linear velocity. A oven temperature programme was done between 50 and 220 ºC with three rates, 5 ºC min^{-1} until 65 ºC, 20 ºC min^{-1} until 185 ºC, and 35 ºC min^{-1} until 220 ºC, and held 1 min at 220 ºC. The detector temperature was at 250 ºC.

External calibration curves were constructed exposing the fibre to a standard mixture of methanol and acetic acid, for concentrations between 40 and 100 mg L^{-1} for methanol and between 25 and 105 mg L^{-1} for acetic acid. These concentration ranges were chosen according to the minimum and maximum content of methanol and acetic acid expected to find in plant polysaccharides. Calibration curves of methanol were also constructed in the presence of two concentrations of acetic acid, 80 and 105 mg L^{-1}, and calibration curves of acetic acid were constructed in the presence of 60 and 100 mg L^{-1} of methanol.

All measurements were made with at least three replicates and the reproducibility was expressed as relative standard deviation (RDS, %). Blanks were run in between each set of experiments.

2.4. Determination of methanol and acetic acid by direct injection of the aqueous solution in the GC-FID

For comparative purposes and validation of the SPME methodology, methanol and acetic acid were also determined by direct injection of aqueous solution in GC-FID. An internal standard, 0.2 mL of 1-propanol, was also added to the vial after saponification and before the total volume of 4.0 mL has been completed with distilled water. The sample suspension was filtered through a glass fibre filter (Whatman GF/C) and a nylon membrane filter NL16 0.2 μm (S&S, Germany). The filtrate was injected (0.5 μL) in a GC HP 5890 series II with a FID detector under the conditions described in 2.3. Calibration curves were constructed for final concentration from 20 to 500 mg L^{-1} for both analytes. Estimated concentrations were made by peak area comparisons with the area of the known concentration of 1-propanol. The samples were prepared in duplicate and each run in duplicate (n=4).

2.5. Carbohydrate analysis

Neutral sugars were obtained by sulfuric acid hydrolysis (Selvendran, March, & Ring, 1979) and analyzed after conversion to their alditol acetates by GC, using 2-deoxyglucose as internal standard (Coimbra, Delgadillo, Waldron, & Selvendran, 1996). A Carlo Erba 6000 GC apparatus with split injector and a FID detector was used, with a 30 m column DB-225 (J&W) with i.d. and film thickness of 0.25 mm and 0.15 μm, respectively. The oven temperature program used was: initial temperature 200 ºC, a rise in temperature at a rate of 40 ºC min^{-1} until 220 ºC and then 220 ºC for 14 min, followed by a rate of 20 ºC min^{-1} until 230 ºC and maintain this temperature for 1 min. The injector and detector temperatures were, respectively, 220 and 230 ºC. The flow rate of the carrier gas (H_2) was set at 1 mL min^{-1}.

Uronic acids (UrAc) were quantified by 3-phenylphenol colorimetric method, as described by Coimbra et al. (1996).

3. Results and discussion

3.1. Optimization of HS-SPME-GC-FID methodology

In this study, two types of SPME fibre coatings were compared for their ability to extract methanol and acetic acid from the headspace.

PDMS fibre is a liquid coating recommended by the manufacturer for absorption of low molecular weight and non-polar volatile compounds (M_w 60–275 Da). However, as PDMS 100 μm is the thickest coat available for SPME, its thick polymer coat also allowed it to be successfully applied in microextraction of polar volatile compounds (Kataoka et al., 2000; Mester et al., 2001). DVB/Carboxen/PDMS is a mixed coating fibre used for a wide range of analytes (volatile, semivolatile and polar compounds - $C_{3–20}$, M_w 40-275 Da). It is reported as most sensitive for small compounds and organic acids due to its increase retention capacity resultant from the mutually potentiating effect of adsorptio and absortion to the stationary phase. The small pores (10 Å on average) of Carboxen make this coating fibre particularly effective for extracting small molecules (Kataoka et al., 2000).

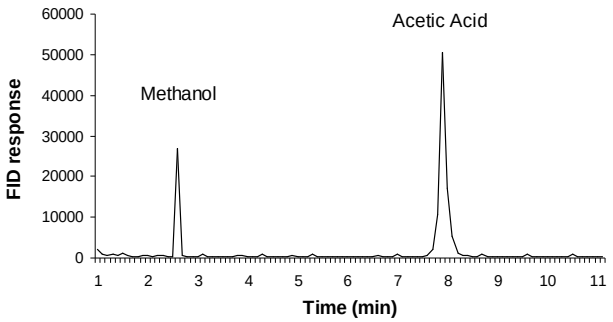

Fig. 1. HS-SPME-GC-FID chromatogram for separation of methanol (2.5 min) and acetic acid (7.8 min).

In the case of the HS-SPME in a three phase system, the amount of an analyte sorbed on the fibre, and the resulting sensitivity, are determined both by sorption kinetics and by the distribution coefficient of the compound between the coating fibre, the headspace and the sample. SPME is, however, sensitive to experimental conditions. Any change in

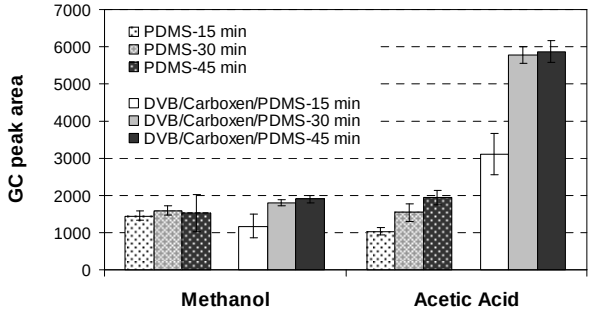

Fig. 2. GC peak areas for methanol and acetic acid using PDMS and DVB/Carboxen/PDMS fibre coatings at three different times of extraction. Solution contained 40 g L^{-1} methanol and 52 g L^{-1} acetic acid at pH 2.0

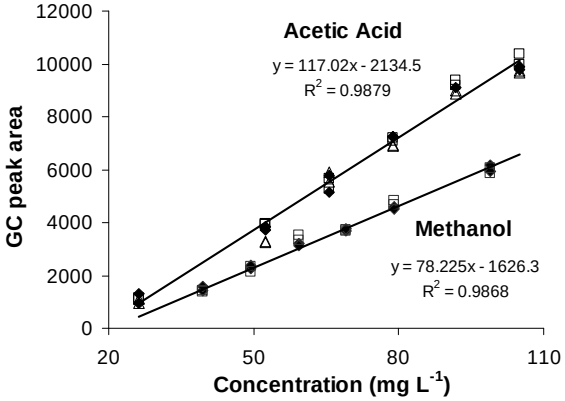

Fig. 3. Calibration curves for methanol with: no acetic acid (♦), 80 mg L^{-1} acetic acid (□) and 105 mg L^{-1} acetic acid (△), and for acetic acid with: no methanol (♦), 60 mg L^{-1} methanol (□) and 100 mg L^{-1} methanol (△).

Table 1
Slope, intercept and r-squared values for calibration curves of methanol and acetic acid by HS-SPME-GC-FID

Calibration curves	Slope	Intercept	*r*-squared
Methanol			
0 μg mL^{-1} acetic acid	75.9 ± 1.6[a]	-1449 ± 108[a]	0.996
80 μg mL^{-1} acetic acid	76.5 ± 3.3[a]	-1464 ± 228[a]	0.981
105 μg mL^{-1} acetic acid	82.3 ± 2.5[a]	-1964 ± 175[a]	0.991
Total data for methanol	78.2 ± 1.6[b]	-1626 ± 107[b]	0.987
Acetic acid			
0 μg mL^{-1} methanol	114.9 ± 3.6[a]	-1993 ± 265[a]	0.991
60 μg mL^{-1} methanol	119.3 ± 3.3[a]	-2161 ± 249[a]	0.992
100 μg mL^{-1} methanol	116.7 ± 4.6[a]	-2243 ± 342[a]	0.985
Total data for acetic acid	117.0 ± 2.3[b]	-2134 ± 167[b]	0.988

[a]Standard deviation (n=18).

[b]Standard deviation (n=54).

experimental parameters, which affects the distribution coefficient and the sorption rate, will influence the amount sorbed and the corresponding reproducibility and sensitivity of the method (Yang & Peppard, 1994). Then, the time required for the extraction of the analytes from headspace was also evaluated for both coatings.

Independently of the type of fibre coating, time of extraction and concentration of analytes, high chromatographic resolution for methanol and acetic acid was observed in the 11 min temperature program. Methanol eluted near 2.5 min (65 ºC) while acetic acid eluted near 7.8 min (165 ºC), as is shown in Fig. 1. Fig. 2 shows the GC peak areas for methanol and acetic acid using both types of SPME coatings with different time of extraction. For acetic acid the areas were three times higher using the DVB/Carboxen/PDMS fibre than the PDMS, while for methanol the areas obtained with the two coatings were similar. The reproducibility obtained for both analytes, shown by the error bars in Fig. 2, is better for times of extraction higher than 30 min when DVB/Carboxen/PDMS fibre was used, revealing that DVB/Carboxen/PDMS fibre is a better coating for methanol and acetic acid extraction than PDMS.

Using DVB/Carboxen/PDMS fibre, the areas for both analytes increased almost twice with an increase from 15 to 30 min of the extraction time, while no significant changes were observed with increasing the time of extraction to 45 min. These results allow to infer that the equilibrium of the amount of compounds in the sample and in the fibre coating was reached, at least, in 30 min. Therefore, 30 min of extraction at 40 ºC with DVB/Carboxen/PDMS fibre coating was chosen as the optimal experimental conditions for the analysis of methanol and acetic acid by HS-SPME.

3.2. Calibration curves of methanol and acetic acid by HS-SPME-GC-FID

External calibration curves for quantification of methanol and acetic acid were performed adding the standards in the concentration range between 40 and 100 mg L^{-1} for methanol and between 25 and 105 mg L^{-1} for acetic acid. The concentrations used were those expected to occur in the polysaccharide samples. The solution matrix waschosen to have the same composition of those containing the saponified

Table 2
Sugars composition of polysaccharides samples from plums and methanol and acetic acid released from them (mg g^{-1} of polysaccharide sample), determined by HS-SPME-GC-FID and by direct injection of the aqueous solution in the GC-FID

Polysaccharide sample	Sugars composition (mol%)								Total sugars (mg g^{-1})	HS-SPME-GC-FID[a]		Direct injection-GC-FID[b]	
	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UrAc		Methanol (mg g^{-1})	Acetic acid (mg g^{-1})	Methanol (mg g^{-1})	Acetic acid (mg g^{-1})
Pulp water washing													
PW	2	0	13	2	5	7	27	45	653	40.0 (3)	–	37.5 (7)	–
AIR													
AIR1	1	1	16	3	2	10	25	42	687	20.5 (6)	11.0 (11)	22.2 (8)	13.8 (10)
AIR2	2	1	14	4	3	12	31	32	618	21.8 (2)	13.1 (10)	22.4 (8)	15.4 (10)
AIR3	2	0	10	4	3	12	42	28	605	15.6 (7)	13.8 (10)	17.3 (7)	15.2 (10)
AIR4	2	1	14	5	2	17	20	41	818	27.5 (7)	7.3 (8)	28.0 (1)	9.3 (10)
AIR water extract													
W1	1	1	13	4	3	7	33	38	562	14.6 (6)	12.4 (5)	13.9 (6)	15.1 (11)
W2	1	0	5	2	5	3	67	15	635	9.2 (11)	10.0 (9)	8.5 (3)	10.3 (11)
W3	1	0	4	1	3	3	72	15	678	16.7 (8)	30.5 (7)	15.6 (4)	34.0 (8)
W4	2	1	10	2	1	10	6	69	997	92.0 (8)	16.7 (3)	87.9 (1)	19.5 (11)
AIR imidazole extract													
Im1	1	0	9	2	1	8	2	77	940	98.2 (2)	–	94.5 (6)	–
Im2	2	1	12	2	2	12	3	66	770	60.5 (5)	–	62.5 (7)	–
Im3	1	1	20	1	1	9	2	65	952	69.7 (1)	–	62.9 (9)	–
Im4	2	1	25	1	1	14	2	54	837	56.2 (7)	26.2 (8)	53.1 (2)	24.2 (10)

[a] Numbers in parentheses correspond to the coefficient of variation (%) (n=3).

[b] Numbers in parentheses correspond to the coefficient of variation (%) (n=4).

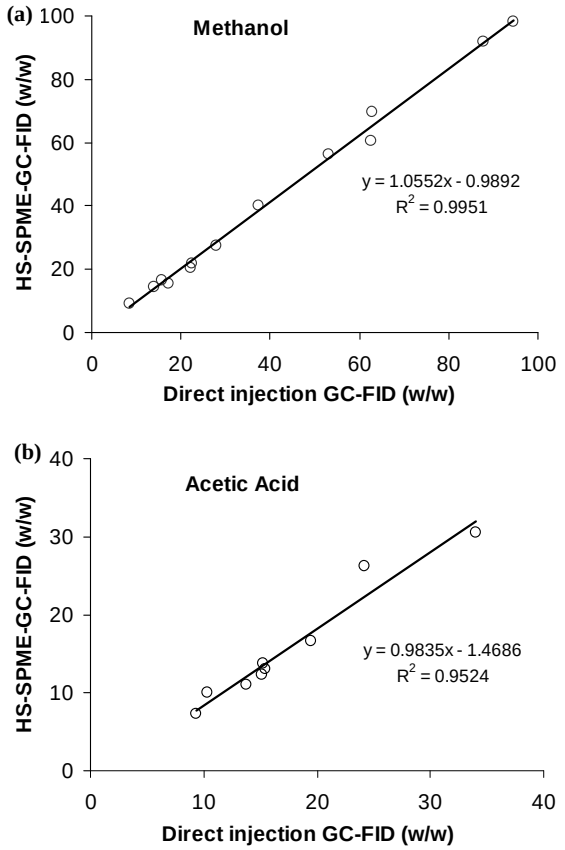

Fig. 4. Comparison of (a) methanol and (b) acetic acid contents (w/w) of polysaccharides, obtained by HS-SPME-GC-FID and by direct injection in GC-FID.

polysaccharide samples, that is, 0.8 mL of NaOH 2 M and 0.8 mL of HCl, pH 2, in a total volume of 4.0 mL.

SPME usually gives a linear response between the concentration of the analyte in solution and the amount of analyte extracted (Kataoka et al., 2000; Mester et al., 2001). However, for porous polymer fibres, like DVB/Carboxen/PDMS, the competitive adsorption process due to the limited surface area available for adsorption may result in the displacement of analytes with lower affinities, affecting therefore the estimation of their concentration (Górecki, Yu, & Pawliszyn, 1999; Mester et al., 2001; Pawliszyn, 2000). In order to evaluate the effect of a possible interference of the variation of the concentration of methanol on the amount estimated of acetic acid and the effect of variation of the concentration of acetic acid on the amount estimated of methanol, calibration curves of each analyte were also constructed in the presence of two different concentrations of the other, 80 and 105 mg L^{-1} of acetic acid and 60 and 100 mg L^{-1} of methanol (Fig. 3). The values of slope, intercept and *r*-squared for the six calibration curves are presented in Table 1. No statistical differences ($p<0.05$) were observed among the three curves obtained for each compound in the presence of the other. These results allowed to conclude that during the extraction procedure with SPME, within this concentration range, no significant competitive interferences occur between these compounds.

The calibration curves used to quantify the methanol and acetic acid content from polysaccharide extracts were obtained using all the values, nine determinations for each concentration (values showed in 'Total' line in Table 1). A linear relationship between the concentration of methanol and acetic acid and their GC peak area was observed, with a *r*-squared values of 0.988 for methanol and 0.987 for acetic acid (Table 1). Based on the standard deviations obtained, the reproducibility of this methodology, expressed as a percentage of the mean of the standard area for each concentration, was estimated to be bellow 10% for both analytes. Quantification limits, determined by the concen-tration of each standard required to give a peak height ten times higher than the noise, was estimated to be 32 mg L^{-1} for methanol and 26 mg L^{-1} for acetic acid.

IV

3.3. Determination of methanol and acetic acid contents of polysaccharides

Methanol and acetic acid contents were determined for a set of plum polysaccharides, obtained from the pulp washing water, from the alcohol insoluble residue (AIR), and from extraction of the AIR with water and 0.5 M imidazole/HCl pH 7.0. These samples differ in their sugar composition (Table 2), namely uronic acids (15–77 mol%) and total amount of sugars (562–997 mg g^{-1}). Methanol and acetic acid content after saponification of samples were determined by HS-SPME-GC-FID and by direct injection of the aqueous solution in the GC-FID, to compare the results, since this methodology has been used for quantification of methanol and acetic acid content of polysaccharides. Statistical significant differences between the two methods for the values of methanol and acetic acid obtained for all polysaccharides analysed were evaluated using *t*-student test at the $p<0.05$ level. No significant differences were observed. The two methodologies can also be compared by the linear relationship for the estimation of both methanol and acetic acid contents (Fig. 4). For methanol, the calculated linear regression had a slope of 1.05±0.05 and a intercept of -2.31±3.21, while for acetic acid the slope was 0.98±0.04 and the intercept was -0.54±0.55. These results show that the calculated slope and intercept for both regression trend lines do not differ significantly from 1 and 0, respectively, and thus there is no evidence for differences between the results obtained by both methods. The reproducibility of the proposed HS-SPME-GC-FID methodology, when applied to the samples, was also shown to be near 10%, a value similar to that found for the standards.

4. Concluding remarks

A simple, rapid and solvent-free methodology for simultaneous determination of methanol and acetic acid released by saponification from plant cell wall polysaccharides is proposed. This methodology comprises the study of the sample headspace by SPME using a DVB/Carboxen/PDMS

coating fibre followed by a GC-FID analysis. For quantitative purposes external calibration curves were used. The results demonstrate a methodology that could be used for analysis of plant cell wall polysaccharides independently of their origin and type, namely, the water insoluble and cellulose-rich AIRs, the highly methylesterified and acetylated pectic polysaccharides of the imidazole extracts or the unpurified water soluble glucan and pectic polysaccharides.

This is a much easier and less time consuming methodology for sample preparation than titration, enzymatic or chemical methods and allows the simultaneous determination of methanol and acetic acid. It is cleaner than the chromatographic methods that require direct injection of acidic aqueous sample solutions. This methodology does not require the use of isotope internal calibration and mass spectrometry, not always available as a laboratory facility. HS-SPME-CG-FID methodology provides a simple, solvent-free, and reproducible procedure, compared to the already established methodologies used for methanol and acetic acid estimation.

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THERMAL AND HIGH-PRESSURE STABILITY OF PURIFIED PECTIN METHYLESTERASE FROM PLUMS (*PRUNUS DOMESTICA*)

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V

ABSTRACT

*Pectin methylesterase (PME) from greengage plums (*Prunus domestica*) has been extracted and purified using affinity chromatography. Only one band on sodium dodecyl sulfateñpolyacrylamide gel electrophoresis was obtained, with an estimated molecular weight of 31 kDa. On isoelectric focusing electrophoresis, two bands with neutral isoelectric points (6.8 and 7.0) were detected. The optimal pH and temperature for plum PME activity were 7.5 and 65C, respectively. A study of purified plum PME thermostability was performed at pH 7.5 and 4.0, indicating a higher thermostability at pH 7.5 than at pH 4.0. A biphasic inactivation behavior was observed for thermal treatments (54-70C), whereas its pressure inactivation could be described by a first-order kinetic model in a pressure range of 650-800 MPa at 25C. Purified plum PME was found to be relatively stable to thermal and pressure (≤600 MPa) treatments, compared to PME from other fruits.*

INTRODUCTION

Greengage plum (*Prunus domestica*) or "Rainha Cláudia Verde" is a Portuguese regional variety. This type of plum can be consumed as fresh fruit,

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but can also be used to obtain the "Ameixa d'Elvas" product. "Ameixa d'Elvas" is the name of the processed plum obtained by a candying process, which has a protected designation of origin (PDO) that produces a natural product with characteristic texture, flavor and color.

As texture is one of the most important characteristics to achieve the desired end-product quality, the study of cell wall polysaccharides and activities of specific cell wall-degrading enzymes is crucial for a better comprehension of the system. Pectic polysaccharides represent a large proportion of the primary cell walls and middle lamella. They contribute to the mechanical strength of the cell wall and to the adhesion between cells; for that reason, pectin is intimately connected to fruit and vegetable firmness (Van Buren 1979).

The structure of the pectic polysaccharides can be altered during fruit ripening or processing caused by the activity of specific cell wall-bound enzymes, mainly pectin methylesterase (PME) and polygalacturonase (PG). PME catalyzes the de-esterification of pectic polysaccharides from cell wall, which can be further hydrolyzed by PG, resulting in a decrease of the degree of polymerization of the pectin chains and a loss of firmness of the tissue. On the other hand, de-esterified pectic polysaccharide chains may cross-link with bivalent ions to form pectate gels, and are less sensitive to β-eliminative breakdown during thermal treatments; these features increase the firmness of the cell wall (Alonso *et al*. 1997a). Therefore, PME can have a beneficial effect on the texture of fruits and vegetables.

PME has already been isolated and characterized from several fruits and vegetables such as peach (Javeri and Wicker 1991), papaya (Fayyaz *et al*. 1995a,b), tomato (Giovane *et al*. 1994), orange (Van den Broeck *et al*. 2000), apple (Denès *et al*. 2000), strawberry (Ly-Nguyen *et al*. 2002a), lemon (Macdonald *et al*. 1993), sweet cherry (Alonso *et al*. 1996), banana (Ly-Nguyen *et al*. 2002b), persimmon (Alonso *et al*. 1997b), grapefruit (Cameron and Grohmann 1995), carrots (Ly-Nguyen *et al*. 2002c; Alonso *et al*. 2003) and green beans (Laats *et al*. 1997). PMEs from different sources have different characteristics, and it is not unusual to find in the same source two or more isoenzymes with different molecular weight (MW), isoelectric points (pIs) and/or kinetic properties.

In this work, PME from plums (*P. domestica*) has been extracted and purified by affinity chromatography. The purified PME was biochemically characterized, and its inactivation behaviour to thermal and high-pressure treatments was evaluated. The objective of this study was to improve the knowledge about plum PME because there is insufficient information available and this enzyme plays a central role in the process of fruit softening. Knowledge about the dependence of plum PME activity and stability on factors such as temperature, pH and pressure will allow us to better understand changes in

plum texture during processing, which could be of great practical importance to improve the processed plum quality.

MATERIALS AND METHODS

Materials

Plums (*P. domestica* cv. "Rainha Claudia Verde") were harvested from a local orchard (Vila Viçosa, Alentejo, Portugal). Apple pectin (degree of esterification [DE] of 75%) was obtained from Fluka Chemicals Co. (Buchs, Switzerland). NHñSepharose 4B resin was purchased from Sigma (St. Louis, MO). All other chemicals were of analytical grade.

PME Purification

The extraction of plum PME was performed according to a slightly modified method described by Denès *et al*. (2000) to prevent loss of activity by phenolic inhibition. The pulp of plums (500 g) was homogenized with the addition of 600-mL distilled water containing 500-mg/L $Na_2S_2O_5$ and 1% polyvinylpolypyrrolidone (PVPP), then centrifuged at 10,000 x g for 30 min. The supernatant was discarded and the pellet was resuspended in 600-mL distilled water containing 500-mg/L $Na_2S_2O_5$ and 1% PVPP, then recentrifuged. The PME was then extracted with 0.2 M tris(hydroxymethyl)aminomethane (Tris) buffer containing 1 M NaCl, 500-mg/L $Na_2S_2O_5$ and 1% PVPP (pH 8.0). After extraction (2 h), the suspension was centrifuged (10,000 x g for 30 min) and the pellet was discarded. This extract was purified by ammonium sulfate precipitation between 30 and 90% saturation. The precipitate was collected by centrifugation (18,000 x g for 15 min) and then dissolved in 20 mM Tris buffer (pH 7.5). This PME crude extract was further purified by affinity chromatography on an NH-Sepharose 4B-PME inhibitor column using the procedure described by Ly-Nguyen *et al*. (2002b).

PME Assay

The PME activity was measured by continuous recording of titration of carboxyl groups released from a pectin solution using an automatic pH STAT (Metrohm, Herisau, Switzerland) and 0.01 N NaOH solution. Assays were performed with a 3.5-mg/mL apple pectin solution (DE of 75%, 30 mL) containing 0.117 M NaCl at pH 7.0 and 22C. One unit of PME activity is defined as the amount of enzyme necessary to generate 1 μmol of carboxyl groups per minute, under the previously mentioned assay conditions.

Protein Determination

Protein concentration was determined using the Sigma Procedure no. TPRO-562 (for kit no. BCA-1 and product no. B-9643). Bovine serum albumin (BSA) (Sigma) was used as the standard.

Gel Electrophoresis

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and isoelectric focusing (IEF) experiments were carried out using a PhastSystem (GE Healthcare, Uppsala, Sweden). SDS-PAGE was performed using PhastGel homogenous 20% and PhastGel Trisñtricine SDS buffer strips. For SDS-PAGE samples, a buffer containing SDS (2.5%) and β-mercaptoethanol (5%) was added before heating in a boiling bath for 5 min. Phosphorylase *b* (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and lactalbumin (14.1 kDa) were used as standard proteins for estimation of MWs. The IEF was performed in a PhastGel IEF media (polyacrylamide gels) (GE Healthcare) with a pH of 3.0-9.0. For the calculation of the pI, a calibration kit (GE Healthcare) containing 11 proteins with pIs ranging between 9.3 and 3.5 was used. Gels were stained with silver nitrate as described by Heuskeshoven and Dernick (1985).

Effect of pH on PME Activity

The effect of pH on the purified plum PME activity was determined using the titrimetrical assay at 22C after adjusting the pH of the reaction solution to the pH values tested (5.5-10.0). Corrections were made to each experiment for the spontaneous de-esterification of pectin under alkaline conditions.

Effect of Temperature on PME Activity

The effect of temperature on the purified plum PME activity was assayed by measuring the production of methanol using a spectrophotometric procedure (Klavons and Bennett 1986). The purified plum PME was mixed with 30 mL of 0.35% pectin in 20 mM Tris buffer (pH 7.5) and 0.117 M NaCl, and the mixture was incubated at a temperature range from 22 to 75C. Corrections were made to each experiment for the spontaneous de-esterification of pectin at elevated temperatures. For each temperature, a time interval was selected where the quantity of methanol versus heating time showed a linear behavior.

The activation energy (E_a) of PME-catalyzed pectin de-esterification was calculated using the Arrhenius equation (Eq. 1):

$$k = k_{ref} \exp\left(\frac{E_a}{R}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right)\right) \quad (1)$$

where k is the rate constant, R is the gas constant (8.314 J/mol/K) and T is the temperature (K).

Thermal Stability and Inactivation Kinetics of the Purified Plum PME

The thermostability of the purified plum PME was screened for 5 min at pH 4.0 and 7.5 after 5 min of heating within a temperature range from 22 to 56C and from 22 to 80C, respectively. The detailed thermal inactivation kinetics was determined between 54 to 70C at pH 7.5. Thermal treatments of the purified plum PME were performed in a temperature-controlled water bath using glass capillaries (Hirschmann, Eberstadt, Germany) to enclose the enzyme solution. After the treatments, the capillaries were immediately cooled in an ice-water bath. The residual activity of the PME was assayed titrimetri-cally within 60 min of storage at 0C, under standard assay conditions described earlier.

Pressure Stability and Inactivation Kinetics of the Purified Plum PME

Pressure treatments were performed in a multivessel high-pressure apparatus (eight vessels of 8 mL) (Resato, Roden, The Netherlands) at pressures ranging from 400 to 800 MPa. The pressure medium is a glycol-oil mixture (TR15; Resato). The purified plum PME was enclosed in 0.3-mL flexible microtubes (Elkay, Leuven, Belgium), and the microtubes were placed in the pressure vessels already equilibrated at 25C. Pressure was built up slowly (100 MPa/min) to minimize temperature increases caused by adiabatic heating. After pressure buildup, an equilibrium period of 2 min to allow temperature to equilibrate to its desired value was taken into account. After 2 min, one pressure vessel was decompressed, the residual activity of the corresponding PME sample was measured and was considered as the blank. The pressure of the other vessel was then released, after 15 min of treatment, for the screening of pressure stability. For kinetic pressure inactivation studies, the other seven vessels, each containing one enzyme sample, were released at predefined time intervals. After pressure release, all samples were immediately cooled in an ice-water bath, and the residual PME activity was measured within 60 min.

Kinetic Data Analysis

A first-order kinetic model can often describe the inactivation of enzymes, whereby enzyme activity decreases log-linearly as a function of time (Eagerman and Rouse 1976):

$$\ln(A / A_0) = k\,t \qquad (2)$$

where A_0 and A are the initial activity and the remaining activity, respectively, at time t. Equation 2 is valid under isothermal and isothermal-isobaric conditions, whereby the inactivation rate constant k can be determined from a linear regression analysis of $\ln(A/A_0)$ versus time.

Enzymes characterized by several isoenzymes present can often be grouped into two fractions, one more resistant to temperature and/or pressure than the other (i.e., labile and stable fractions) and both inactivating according to a first-order decay. The inactivation can be modeled as a biphasic kinetic model (Eq. 3), assuming that the inactivation of both fractions is independent (Chen and Wu 1998; Van den Broeck *et al*. 2000).

$$A = A_L \exp(-k_L\, t) + A_S \exp(-k_S\ t) \tag{3}$$

where k is the first-order inactivation rate constant, and the subscripts L and S means labile and stable enzyme fractions, respectively.

The kinetic parameter values for the thermal inactivation of plum PME were estimated by nonlinear regression analysis of Eq. (4), which is obtained by inserting the Arrhenius model (Eq. 1) into Eq. (3).

$$A = A_L \exp\left(-k_{Lref} \exp\left(\frac{E_{aL}}{R}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right)\right)\cdot t\right) + A_S \exp\left(-k_{Sref} \exp\left(\frac{E_{aS}}{R}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right)\right)\cdot t\right) \tag{4}$$

The pressure dependence of the inactivation rate constants can be expressed by the activation volume using the Eyring equation (Eq. 5):

$$\ln(k) = \ln(k_0) - \left[\frac{V_a}{RT}(P - P_0)\right] \tag{5}$$

where P and P_0 are the absolute pressure (MPa) and the reference pressure (MPa), respectively; k_0 is the rate constant at P_0; V_a is the activation volume (cm^3/mol); and R (8.314 cm^3/MPa/mol/K) is the universal gas constant. An estimate of the activation volume for plum PME inactivation was obtained through linear regression analysis on Eq. (5).

RESULTS AND DISCUSSION

Extraction, Purification and Characterization of Plum PME

Plum PME, as in other fruits, is ionically bound to the cell walls and requires a buffer with suitable high ionic strength (0.2 M Tris buffer, 1 M NaCl

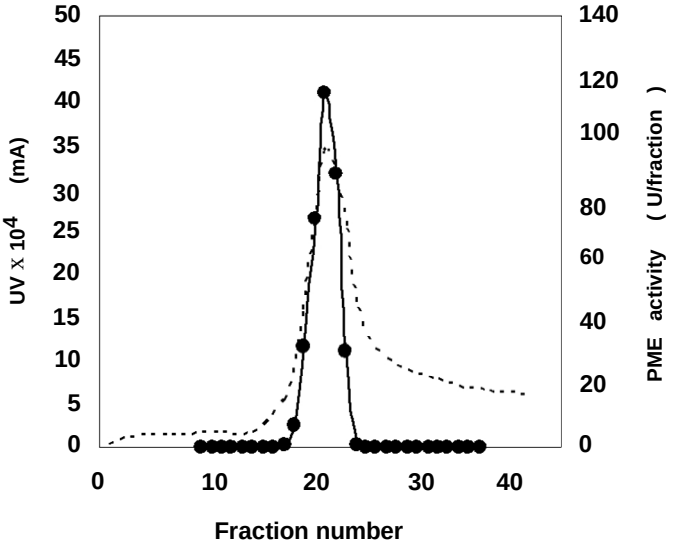

FIG. 1. ELUTION PROFILE OF PLUM PECTIN METHYLESTERASE (PME) ON AN NH-SEPHAROSE 4B (SIGMA, ST. LOUIS, MO)-PME INHIBITOR COLUMN
Volume of each fraction, 2 mL. UV absorbance measured at 280 nm (- - - -); PME activity (●). Washing step is not shown.

[pH 8.0]) for extraction from cell walls (Glover and Brady 1995). Because plums have a high content of phenolic compounds, before PME extraction the pulp had to be washed with a solution containing $Na_2S_2O_5$ and PVPP to prevent PME activity loss by phenolics (Anderson 1968; Salunkhe and Kadam 1995). No PME activity was detected in the supernatants. This procedure prevents any loss of activity by phenolic inhibition and improves the enzyme solubilization from the cell walls (Denès *et al*. 2000).

The plum PME extract was partially purified by ammonium sulfate precipitation, and it requires 90% saturation for the precipitation of PME. Usually, PMEs from other plant sources needed 80% of ammonium sulfate saturation for precipitation (Javeri and Wicker 1991; Alonso *et al*. 1996; Ly-Nguyen *et al*. 2002b), whereas for plum PME at 80% saturation, the PME activity was still detected in the supernatant. It was only when the ammonium sulfate concentration was increased to 90% that no PME activity was detected in the supernatant. A single PME activity and a protein peak were obtained from the affinity column after elution with a high ionic strength and a high pH buffer. Figure 1 shows the chromatographic elution profile of the purified plum PME. Unbound proteins were removed with 2 mM KH_2PO_4 buffer (pH 6.0) with 0.5 M NaCl during the washing step (data not shown). Six extractions and purifications were carried out, and each purification of plum PME from the crude extract produced a single peak.

TABLE 1.

PURIFICATION OF PLUM PECTIN METHYLESTERASE (PME)

	Activity (U)	Protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification factor
Crude extract	3072	193.5	15.9	100	1
Purified PME	1050	1.76	596.6	34.2	37.6

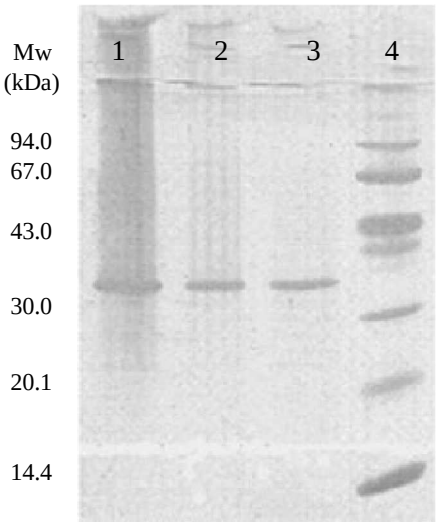

FIG. 2. SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS OF PLUM PECTIN METHYLESTERASE (PME)

Molecular mass standards (lane 1) and purified plum PME (lanes 2 and 3) contain, respectively, one-fourth and one-third of the protein content of lane 4.

A summary of the purification procedure is given in Table 1. The purified plum PME had a specific activity of 596.6 U/mg protein, corresponding to a 37.6-fold enrichment and a recovery percentage of at least 34%, in relation to the total enzymatic activity of the 90% ammonium sulfate precipitate.

The purified plum PME showed only one band on SDS-PAGE (Fig. 2). Based on the electrophoretic mobility of standard proteins and using Image-Master 1D software (GE Healthcare), the MW was estimated as 31 kDa. This molecular mass is in accordance with the PMEs purified from the same species (*Prunus*) of fruits, 27 and 56 kDa for sweet cherry (Alonso *et al*. 1996) and 33.9 and 36.3 kDa for peach (Javeri and Wicker 1991). This value is also within the range of other fruit PMEs: 34.5 and 36 kDa for apple (Denès *et al*. 2000), 33.5 and 43 kDa for strawberry (Ly-Nguyen *et al*. 2002a), 33 and 35 kDa for lemon (MacDonald *et al*. 1993), 30 and 51 kDa for persimmon (Alonso *et al*. 1997b), 20-40 kDa for red grapefruit (Cameron and Grohmann 1995) and 31 kDa for tomato (Giovane *et al*. 1994).

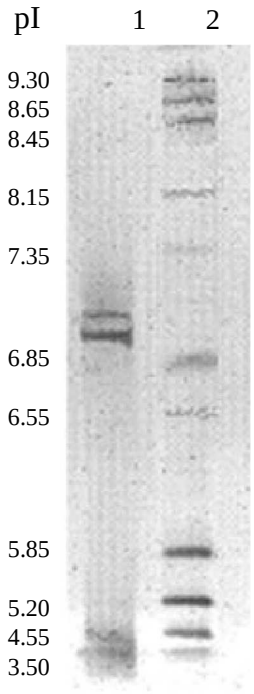

FIG. 3. ISOELECTRIC FOCUSING GEL OF PLUM PECTIN METHYLESTERASE (PME)
Isoelectric point (pI) standards (lane 1) and purified plum PME (lane 2).

Two bands were detected on the IEF gel, which had a neutral pI of 6.8 and 7.0, respectively (Fig. 3). The pIs of plant PMEs are normally reported to be between 6 and 11, as determined for banana (Ly-Nguyen *et al*. 2002b), persimmon (Alonso *et al*. 1997b), sweet cherry (Alonso *et al*. 1996) and pepper (Castro *et al*. 2004) PMEs.

Effect of pH on Purified PME Activity

The effect of pH on PME activity was determined in a range from 5.5 to 10.0 (Fig. 4). The obtained curve revealed a rapid increase of PME activity from pH 5.5-7.0, and a slow decrease between 8.0 and 10.0, with 66% of the maximal activity at pH 10.0. The purified plum PME showed an optimum pH value of 7.5. The optimum pH value obtained, as well as the shape of the curve, is in agreement with the results which have been reported for purified PME from different sources (Javeri and Wicker 1991; Alonso *et al*. 1997b, 2003; Denès *et al*. 2000; Ly-Nguyen *et al*. 2002b; Castro *et al*. 2004). A spontaneous de-esterification of pectin was detected above pH 8.0, and the corrections were made for each pH.

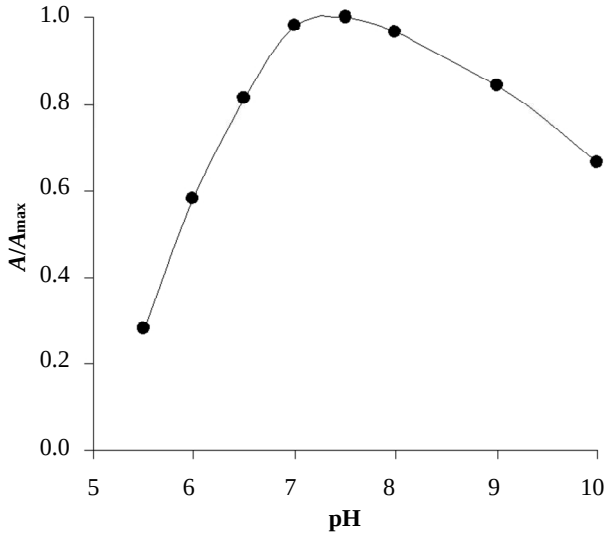

FIG. 4. RELATIVE ACTIVITY OF PLUM PECTIN METHYLESTERASE AS A FUNCTION OF pH (T = 22C)

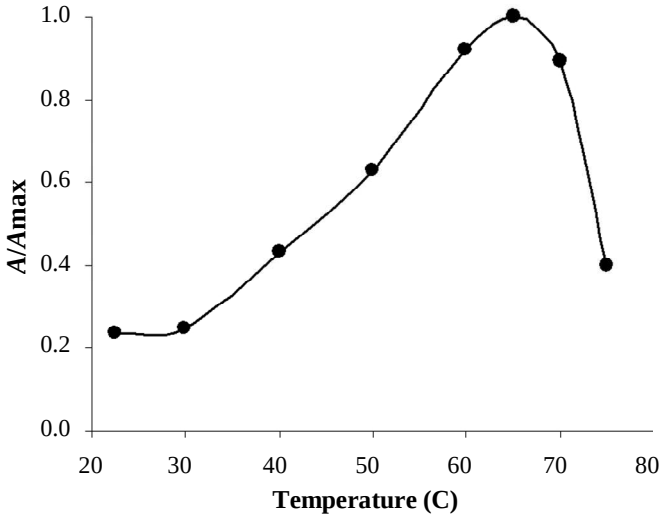

FIG. 5. RELATIVE ACTIVITY OF PLUM PECTIN METHYLESTERASE AS A FUNCTION OF TEMPERATURE (pH 7.5)

Effect of Temperature on Purified PME Activity

The activity of the purified plum PME at pH 7.5 as a function of temperature (22-75C) is depicted in Fig. 5. The maximal PME activity was reached at 65C, and above this value the activity decreases sharply. The optimum temperature value determined for plum PME activity is within the

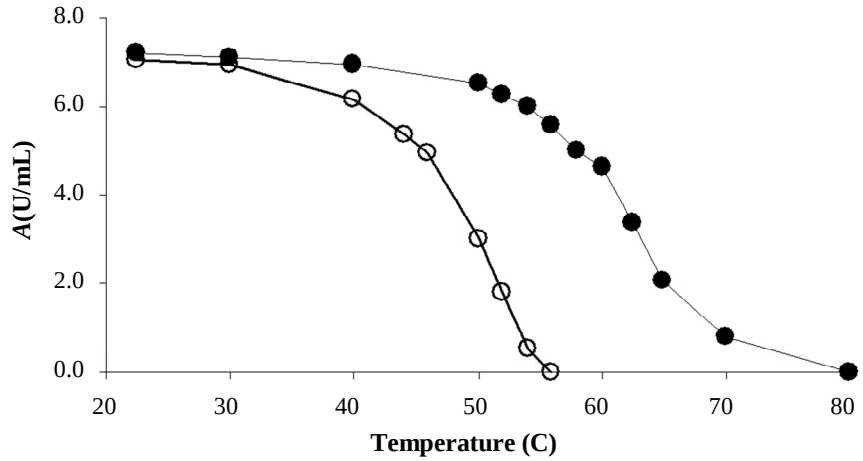

FIG. 6. THERMAL STABILITY (AFTER 5 MIN OF HEATING) OF PURIFIED PLUM PECTIN METHYLESTERASE AT pH 7.5 (-●-) AND 4.0 (-○-)

range of values reported for fruit PMEs such as apple (63C), banana (63C), papaya (65C) and strawberry (60C) (Fayyaz *et al*. 1995a; Denès *et al*. 2000; Ly-Nguyen *et al*. 2002a,b).

The E_a for plum PME activity was determined using the Arrhenius equation between 22 and 65C as 31.1 ± 2.2 kJ/mol. This value is similar to the values of E_a of PME reported for peach (34.6 kJ/mol) and apple (31.2 kJ/mol) by Javeri and Wicker (1991) and Denès *et al*. (2000), respectively.

Thermal Stability of the Purified Plum PME

The thermostability of the purified plum PME (in 20 mM Tris buffer (pH 7.5)) was screened after 5 min of heat treatment at several temperatures (22-80C). Figure 6 shows that plum PME maintained minimally 90% of the initial activity after 5 min of treatment at temperatures below 50C. From 50 to 60C, the PME was gradually inactivated; above 60C, the PME activity rapidly decreased. A treatment at 80C for 5 min completely inactivated the purified plum PME at pH 7.5.

The thermal stability of the purified plum PME was also evaluated at pH 4.0 (20 mM citrate buffer), the pH of plum pulp (Fig. 6). The purified plum PME was much less thermostable at pH 4.0 compared to pH 7.5. At 52C, less than 30% of the initial activity was retained and no PME activity was detected after 5 min of heating at 56C. According to these heat-stability studies, the purified plum PME is less thermostable at pH 4.0 than at pH 7.5, which is in agreement with a previous study performed by Fayyaz *et al*. (1995b) for purified papaya PME.

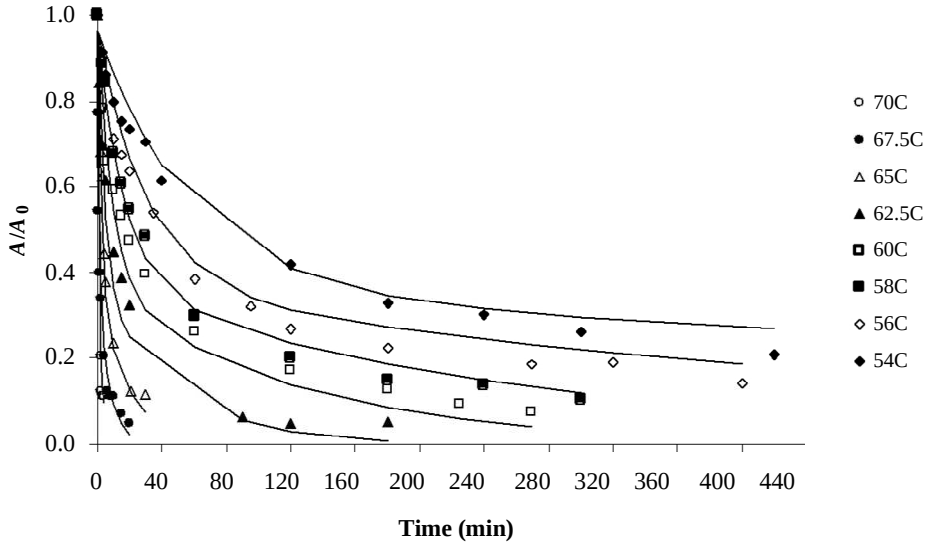

FIG. 7. RELATIVE THERMAL INACTIVATION OF PURIFIED PLUM PECTIN METHYLESTERASE DISSOLVED IN 20 mM TRIS(HYDROXYMETHYL)AMINOMETHANE BUFFER AT pH 7.5 AND ATMOSPHERIC PRESSURE, MODELED USING A BIPHASIC INACTIVATION MODEL

Thermal Inactivation Kinetics of the Purified Plum PME

A detailed kinetic study of the isothermal inactivation at atmospheric pressure of the purified plum PME (20 mM Tris buffer, pH 7.5) was performed from 54 to 70C (Fig. 7). Isothermal inactivation could be described accurately by a biphasic model, which indicates the presence of a heat-labile and a heat-resistant fraction in purified plum PME. The presence of labile and resistant fractions of PME has been reported in other PMEs purified from several fruits and vegetables such as oranges (Van den Broeck *et al.* 2000), white grapefruits (Seymour *et al.* 1991), sweet cherries (Alonso *et al.* 1996), papaya (Fayyaz *et al.* 1995b), persimmon (Alonso *et al.* 1997b), green beans (Laats *et al.* 1997) and pepper (Castro *et al.* 2004).

The relative activity, inactivation rate constants and activation energies for both fractions were estimated using nonlinear regression analysis on Eq. (4) (Table 2). The purified plum PME thermostability is similar to the one reported for purified banana PME by Ly-Nguyen *et al.* (2002b). Although when compared with PMEs from other plant sources, it seems to be more thermostable. For example, at 60C, the inactivation rate constant estimated for the labile fraction was 0.106 per minute, and for the stable one 0.008 per minute, while for pepper PME higher values of 0.8084 and 0.0107 per minute were reported (Castro *et al.* 2004). The thermolabile plum PME fraction contributed to about 60% of the total PME activity. The E_a for the heat-labile and heat-resistant forms, under the temperature range studied

TABLE 2.
KINETIC PARAMETERS FOR THERMAL INACTIVATION OF PURIFIED PLUM PECTIN METHYLESTERASE DISSOLVED IN 20 mM TRIS(HYDROXYMETHYL)AMINOMETHANE BUFFER AT pH 7.5, MODELED USING A BIPHASIC INACTIVATION MODEL

Labile fraction	A_L (%)	59.1± 3.4*
	k_{L60C} (per minute)	0.106± 0.011
	E_{aL} (kJ/mol)	273.9± 8.9
Stable fraction	A_S (%)	37.0± 3.5
	k_{S60C} (per minute)	0.008± 0.001
	E_{aS} (kJ/mol)	354.3± 16.5

* SE of regression.

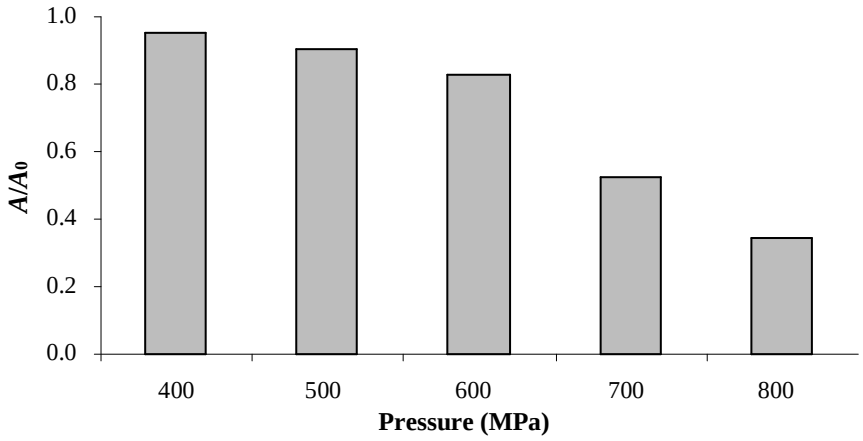

FIG. 8. RELATIVE PRESSURE STABILITY OF PURIFIED PLUM PECTIN METHYLESTERASE (PME) AT 25C (*A* IS PME ACTIVITY FOR TREATED SAMPLES AND A_0 IS PME ACTIVITY FOR UNTREATED SAMPLES)

were, respectively, 273.9 ± 8.9 and 354.3 ± 16.5 kJ/mol. The E_a for the thermostable fraction is comparable with the ones described for commercial orange PME (301.4-350.5 kJ/mol) by Van den Broeck *et al*. (1999) and green beans PME (305-330 kJ/mol) by Laats *et al*. (1997).

Pressure Stability of the Purified Plum PME

The purified plum PME has been screened for its pressure stability at 25C and pH 7.5 by pressurizing the samples for 15 min. Figure 8 illustrates the results of pressure resistance assays carried out in the range of 400-800 MPa. The purified plum PME was very stable to pressure treatments below 600 MPa. For pressures up to 800 MPa, a decrease in the relative residual

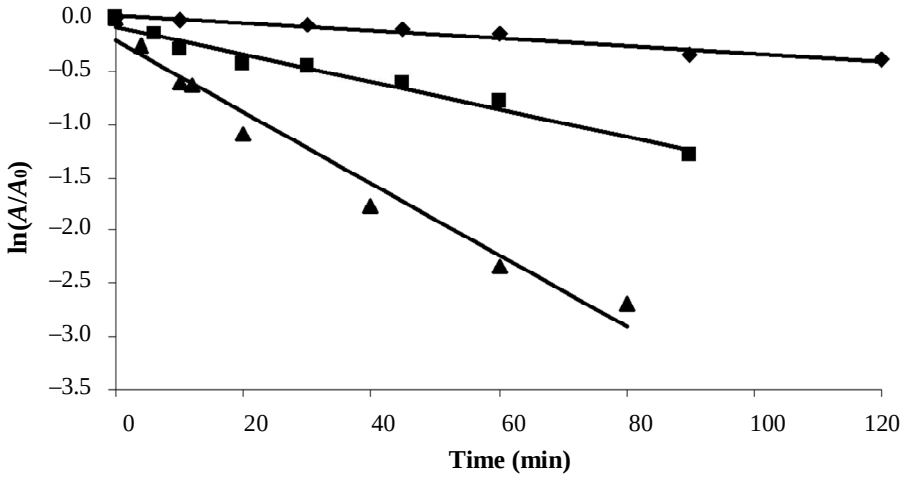

FIG. 9. RELATIVE HIGH-PRESSURE INACTIVATION AT 25C OF PURIFIED PLUM PECTIN METHYLESTERASE (PME) DISSOLVED IN 20 mM TRIS(HYDROXYMETHYL)AMINOMETHANE BUFFER AT pH 7.5 AT 650 (♦), 725 (■) AND 800 MPa (▲)

A is PME activity for pressure-treated samples and A_0 is PME activity for untreated samples.

activity of the PME was observed, with a maximum loss of 50% of the initial activity. Like other PMEs purified from different sources, the plum PME was revealed to be very pressure resistant for short treatments below 600 MPa, at room temperature (Fachin *et al*. 2002; Castro *et al*. 2004).

High-pressure Inactivation Kinetics of the Purified Plum PME

Based on the pressure-stability studies, a kinetic study of pressure inactivation has been performed in the range from 650 to 800 MPa at 25C. The high-pressure inactivation of the purified plum PME dissolved in 20 mM Tris buffer (pH 7.5) could be adequately described by a first-order model in a pressure range of 650-800 MPa (Fig. 9). The inactivation rate constants, estimated using linear regression analysis of $\ln(A/A_0)$ versus time, are reported in Table 3. The inactivation rate constants increased with increasing pressure levels applied. The pressure dependence of the inactivation rate constants of the purified plum PME within the pressure range was estimated by linear regression analysis as -36.80 ± 3.2 cm^3/mol ($r^2 = 0.993$) using the Eyring relationship. The absolute value estimated for the activation volume of the purified plum PME at 25C is lower than that obtained for banana PME (−59.2 cm^3/mol) by Ly-Nguyen *et al*. (2002b), but higher to those values reported for orange PME (−24.55-29.29 cm^3/mol) and strawberry PME (−10.77 cm^3/mol) (Van den Broeck *et al*. 2000; Ly-Nguyen *et al*. 2002a).

TABLE 3.
HIGH-PRESSURE INACTIVATION KINETIC PARAMETERS ESTIMATED FOR PURIFIED PLUM PECTIN METHYLESTERASE DISSOLVED IN 20 mM TRIS(HYDROXYMETHYL)AMINOMETHANE BUFFER AT pH 7.5 AND AT 25C

Pressure (MPa)	*k* (/min)	r^2
650.0	0.0036 ± 0.0004*	0.95
725.0	0.0130 ± 0.0009	0.97
800.0	0.0336 ± 0.0024	0.97

* SE of regression.

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Characterization of plums procyanidins by thiolytic depolymerization

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ABSTRACT

The phenolic compounds of 'Green Gage' (GG) plums (Prunus domestica L.), 'Rainha Cláudia Verde', from a "Protected Designation of Origin" (PDO), in Portugal, were quantified in both flesh and skin tissues of plums collected in two different orchards (GG-V and GG-C). Analyzes of phenolic compounds were also performed on another GG European plum obtained in France (GG-F), and two other French plums, 'Mirabelle' (M) and 'Golden Japan' (GJ). Thiolysis was used for the first time in the analysis of plums phenolic compounds. This methodology showed that the flesh and the skin contain a large proportion of flavan-3-ols, which account, respectively, for 92 and 85% in GJ, 61 and 44% in GG-V, 62 and 48% in GG-C, 54 and 27% in M, and 45 and 37% in GG-F. Terminal units of procyanidins observed in plums are mainly (+)-catechin (54-77% of all terminal units in flesh and 57-81% in skin). The GJ plums showed a phenolic composition different from all the others, with a lower content of chlorogenic acids isomers and the presence of A-type procyanidins as dimers and as terminal residues of polymerized forms. The average degree of polymerization (DPn) of plum procyanidins was higher in the flesh (5-9 units) than in the skin (4-6 units). Procyanidin B7, which was never reported to occur in plums, was observed in the flesh of all GG plums and in the skin of the Portuguese ones. Principal component analysis of the phenolic composition of the flesh and the skin of these plums obtained after thiolysis allowed their distinction according to the variety and origin, opening the possibility of the use of phenolic composition for variety/origin identification.

KEYWORDS: 'Green Gage'; 'Mirabelle'; 'Golden Japan'; reversed-phase HPLC; phenolic compounds; hydroxycinnamic acid; flavan-3-ols; flavonols

INTRODUCTION

Phenolic compounds are relevant for the establishment of fruits quality, as they play a role in the visual appearance (pigmentation and browning), taste (astringency), and health-promoting properties (free-radical scavengers) of fresh fruits and their derived products (*1-3*). Some of the health benefits related to fruit consumption seem to be due to the content of phenolic compounds with antioxidant, anticarcinogenic, antimicrobial, antiallergic, antimutagenic, and anti-inflammatory activities (*1, 2, 4*). As a general rule, accumulation of soluble phenolic compounds in fruits is greater in the outer tissues (skin) than in the inner tissues (flesh) (*1, 5*). The level and composition of phenolic compounds in plums are of great importance for some of the health promoting benefits attributed to the consumption of these fruits. For instance, studies measuring the total antioxidant capacity of a number of fruits revealed that plums showed one of the highest antioxidant activities within Mediterranean fruits (*2, 6-9*). Among the molecules exerting antioxidant activity in plums, the contribution of phenolic compounds was found to be much greater than vitamin C and carotenoids (*2, 7, 10-12*).

The predominant class of phenolic compounds identified in plums was the hydroxycinnamic acid derivatives such as 3-caffeoylquinic acid (neochlorogenic acid) and 5-caffeoylquinic acid (chlorogenic acid), the former being present in higher amounts (*13-15*). Flavan-3-ols were also identified in plums, predominantly the procyanidin dimers of the B- and A-types, as well as procyanidin trimers and monomers (*13*). Rutin (quercetin 3-rutinoside) was the principal polyphenol among the flavonol glycosides found in plums (*13*). However, no information is yet available about the total concentration and degree of polymerization of plum procyanidins.

The presence of flavan-3-ol monomers and procyanidins in the phenolic composition of the fruits is of great importance for their nutritional and functional properties. The monomers, which are good substrates for polyphenol oxidase, are directly involved in enzymatic oxidation. Conversely, procyanidins could be involved in oxidation reactions (*16*). Flavan-3-ols could have several physiological roles, as protective against fungi attacks and biological properties, being these properties directly correlated with the

molecular weight of procyanidins (*17, 18, 19*). A-type dimers have been reported as having antibacterial activity (*14*).

Protected designation of origin (PDO) is an official label used in the European Union for food products which are produced, processed, and prepared in a given geographical area, due to distinctive quality and organoleptic characteristics. In spite of this, scientific information available to support the unique quality of many PDO products, although essential, is still very scarce, as is the case of the "Ameixa d'Elvas" plum.

"Ameixa d'Elvas" is a PDO for a plum (*Prunus domestica* L.) of a specific type of 'Green Gage' variety, "Rainha Cláudia Verde", produced in a defined region in Alto Alentejo (South-East of Portugal). The aim of this work was to characterize these plums by their phenolic composition, for possible further use of the required information to unambiguously identify these plums. For this objective the phenolic composition of PDO 'Green Gage' plums from Portugal (collected in two different orchards from the same limited geographic area within the PDO) was studied together with the plums of the same variety but from a different geographic origin (France), and also with plums of other variety and specie ('Mirabelle' and 'Golden Japan'), whose phenolic composition has not yet been established. Reversed-Phase HPLC (RP-HPLC) analyses were performed on crude methanolic extracts from both the skin and the flesh. Additionally, RP-HPLC were also carried out after thiolysis on freeze-dried materials of skin and flesh to obtain more information on the total procyanidins content with global characterization of their average degree of polymerization and their constitutive flavanol units.

MATERIALS AND METHODS

Solvents and Reagents. Methanol, formic acid, and acetonitrile of chromatographic grade quality and glacial acetic acid were purchased from Biosolve Ltd (Netherlands). Folin-Ciocalteu reagent was purchased from Merck (Germany). Deionized water was obtained with a Milli-Q water system (Millipore Corporation, Bedford, MA).

Phenolic Standards. (+)-Catechin, (-)-epicatechin, 5-caffeoylquinic acid, and rutin were provided by Sigma-Aldrich Inc. (Bellefonte, PA, USA). Hyperoside (quercetin 3-galactoside), isoquercitrin (quercetin 3-glucoside), quercitrin (quercetin 3-rhamnoside), myricetin, and isorhamnetin rutinoside were provided by Extrasynthese S.A. (France). (-)-Epicatechin benzylthioether was obtained as described by Guyot *et al*. (*20*). Procyanidin A2 was kindly provided by E. Meudec (UMR-SPO, INRA, Montpellier, France). Procyanidins B1, B2, and B7 were available at INRA, UR117 (Le Rheu, France) as standards isolated from apple for B2, or by hemi-synthesis for B1 and B7, according to a method adapted from previously published results of Hemingway and McGraw (*21*).

Samples. Plums (*Prunus domestica* L.) of 'Green Gage' variety were harvested at mature stage in 2006 in two orchards from Alto Alentejo, Portugal ("Cano" – GG-C and "Vila Viçosa" – GG-V). The analyzes were also performed on three varieties of French plums, 'Green Gage' (GG-F) (*Prunus domestica* L.), 'Mirabelle' (M) (*Prunus domestica* L.), and 'Golden Japan' (GJ) (*Prunus salicina* Lindl.) obtained in a local market in Rennes (France). All plums were brought to the laboratory and immediately prepared for analysis.

Plums (1 kg) were peeled and the flesh and skin were separately immersed in a NaF solution (to inhibit oxidation) at 1 g/L in water, with a proportion of 2 g of fresh material for 3 mL of aqueous solution and homogenized in a Waring blender for 1 min. The homogenized samples were immediately frozen and freeze-dried.

Extraction of phenolic compounds. Phenolic compounds were extracted from freeze-dried plum samples (0.1 g) with 1 mL methanol (*20*) containing 1% acetic acid (to avoid oxidation) using an ultrasound-assisted method (*22*) for 15 min, at room temperature. This crude methanol extract was filtered through a 0.45 μm filter (HV, Millipore Corporation, Bedford, MA) and immediately analyzed. Three extractions were carried out for each sample.

Quantification of Total Phenolic Compounds. Total phenolic compounds were quantified by the colorimetric method of Folin-Ciocalteu (*23*) adapted from Guyot *et al.*

(*20*). The crude methanol extract was properly diluted with acetic acid (2% v/v in water) and 0.5 mL of extract was added to 250 μL of Folin-Ciocalteu reagent. This procedure, when applied to plums, according to Chun and Kim (*24*), does not require correction for vitamin C or reducing sugars content. 5-Caffeoylquinic acid and (-)-epicatechin were both used as standards. The calibration curves were done with 7 different concentrations and each one was measured in triplicate. Quantifications of phenolic compounds were obtained by reporting the absorbances in both calibration curves of 5-caffeoylquinic acid (0.02-0.12 mg/mL) and (-)-epicatechin (0.02-0.17 mg/mL). Phenolic compounds in each extract were quantified in triplicate.

Thiolysis of the samples. The thiolysis of plums flesh and skin was performed on 50 mg of homogeneous freeze-dried powder with 800 μL of benzylthiolether (5% v/v in dry methanol) and 400 μL of 0.4 M HCl in dry methanol (*25*) and the reaction was carried out at 40 °C during 30 min. The samples were filtered through a 0.45 μm Teflon membrane (Millipore, Bedford, MA) and then analyzed. Thiolysis reactions were performed in triplicate for each sample.

Reversed-Phase HPLC. The crude methanol extracts and the thiolysis reaction mixtures were analyzed by reversed-phase HPLC (RP-HPLC). The HPLC apparatus was a Waters (Milford, MA, USA) system, equipped with an autosampler 717, with a cooling module set at 4 ºC, a 600 E multisolvent pump delivery system, and a 996 photodiode array detector. The data acquisition and remote control of the HPLC system was done by the Millennium 32 version 3.20 software. The column was a Fusion-RP, polar embedded C18 (Synergi, Phenomenex Inc, USA) and the eluents used were 1% formic acid in water (solvent A) and acetonitrile (solvent B), degassed continuously with helium. The flow rate was 1 mL/min and the injection volume was 5 μL. The solvent gradient started with 95% A; 0-34 min, 60% A linear; 34-37 min, 10% A linear; 37-42 min, 10% A isocratic; 42-47 min, 95% A linear and return to initial conditions. The column oven was set at 35 ºC. The eluate was continuously monitored between 190 and 600 nm. Phenolic compounds in the crude extracts and thiolysed samples were quantified in triplicate.

RP-HPLC characterization and quantification of phenolic compounds. Phenolic compounds were identified on chromatograms by comparison of their retention times with available standards and UV-visible spectra: caffeic acid, (+)-catechin, 5-caffeoylquinic acid, *p*-coumaric acid, *p*-coumaroylquinic acid, (-)-epicatechin, (-)-epicatechin benzylthioether, ferulic acid, hyperoside (quercetin 3-galactoside), isoquercitrin (quercetin 3-glucoside), isorhamnetin rutinoside, kaempferol glucoside, kaempferol rutinoside, myricetin, phloretin, procyanidins A, B1, B2, B5 and B7, quercitin, quercitrin (quercetin 3-rhamnoside) and rutin. Due to the unavailability of authentic commercial 3-caffeoylquinic acid standard, its identification was accomplished by comparison of a previously reported HPLC separation pattern and of its UV-Vis spectrum with that of 5-caffeoylquinic acid (*13, 14, 26*). Although other compounds could not be completely identified, they were characterized according to their class on the basis of their UV-visible spectra (*27*): flavan-3-ols (monomers and procyanidins) by the presence of a single symmetric band with a maximum absorbance at 278 nm, hydroxycinnamic acid derivatives that presented a maximum absorbance between 300 and 330 nm, and flavonols that showed an absorption band with a maximum above 340 nm.

Quantification of the identified compounds was performed using external calibration curves obtained for (+)-catechin (0.01-0.14 mg/mL), (-)-epicatechin (0.02-0.10 mg/mL), 5-caffeoylquinic acid (0.02-0.9 mg/mL), rutin (0.02-0.2 mg/mL), and (-)-epicatechin benzylthioether (0.02-0.5 mg/mL). The quantification of hydroxycinnamic acid derivatives was evaluated at 320 nm applying the 5-caffeoylquinic acid calibration curve. The flavonols were quantified at 350 nm using rutin as external standard.

The average degree of polymerization (DPn) of flavanols (including monomeric catechins plus procyanidins) was calculated as previously published for apple samples (*20*). For ‘Golden Japan’ variety that contained A-type moieties in the procyanidin structure, the DPn was calculated by taking into account A-type dimers formed in thiolysis media according to the formula given by Le Roux *et al.* (*28*).

HPLC-ESI-MS. HPLC-ESI-MS was used for unambiguous detection of phenolic compounds in the samples, to eliminate misidentification of coeluting compounds with similar UV spectra. The mass spectrometry (MS) system was an LCQ Deca ion trap

(Thermofinnigan, San Jose, CA, USA) equipped with an electrospray ionization (ESI) source. ESI-MS was coupled to an HPLC system consisting of a degasser spectra system SMC 1000 (Thermofinnigan, San Jose, CA, USA), a HP quaternary gradient Pump 1100 series (Agilent Technologies), an autosampler Surveyor (Thermofinnigan, San Jose, CA, USA), a Zorbax Eclipse XDBC18 column (2,1 cm x 150 mm), a DAD spectra system UV 6000 LP (Thermofinnigan, San Jose, CA, USA), and Xcalibur® version 1.2 software to monitor the system and to acquire and process the data. The crude extracts and thiolysed samples were injected onto the HPLC-ESI-MS system. The eluting conditions were as follow: solvent A (0.1% formic acid in purified water), solvent B (acetonitrile with 0.1% formic acid), the flow was set at 0.2 mL/min, and the applied gradient was 3% B; 0-5 min, 9% B linear; 5-15 min, 16% B linear; 15-45 min, 50% B, then 5 min washing with 90 % of B an return to initial condition for equilibration. The mass spectrometer was used in the negative mode and the source parameters were: spray voltage of 4500 kV, -60 V orifice voltage, 225 ºC capillary temperature, a 50 arbitrary units sheath nitrogen gas flow rate, and ions were detected in the *m*/*z* 50-2000 range.

Principal Component Analysis (PCA). PCA (*29*) was applied to the compounds identified before thiolysis for hydroxycinnamic acid and flavonols and after thiolysis for flavan-3-ols using the values on a mg/kg fresh weight basis given in **Tables 2** and **3**. The flesh data matrix comprised 5 samples of the plums flesh (3 repetitions) and 12 phenolic parameters, giving a matrix with 15 rows and 12 columns. For skin data, the same plum samples were used but this time 18 phenolic parameters were measured, thus given a matrix with 15 rows and 18 columns. The only pre-processing method used was centering the matrices by column before PCA.

Statistical analysis. Results are presented as mean values and the reproducibility of the results was expressed as standard deviation in tables and as error bars in figures. Statistical analysis between experimental results was based on Student's *t* test. Significant difference was statistically considered at the level of $p<0.05$.

RESULTS AND DISCUSSION

Two different tissue zones of the plums were considered for the analysis of their polyphenolic content, the epidermis (skin) and the parenchyma (flesh). The fruits analyzed had different average weight and the relative amount of skin and flesh was also different, as showed in **Table 1**. The GJ plums were bigger (55 g) and had the highest amount of skin (25%) when compared to the other plums, while M variety had the smallest fruits (10 g) and the lowest amount of skin (11%). The weight of GG varied from 26 g (GG-F) to 38 g (GG-V) and the skin amount ranged from 16-18%.

Table 1. Average fresh weight of intact plums and relative proportion of the flesh, skin and stone.

Plum varieties	*Weight (g)*	*Flesh (%)*	*Flesh Moisture (%)*	*Skin (%)*	*Skin Moisture (%)*	*Stone (%)*
GG-V	38 ± 3	74	83 ± 1	16	76 ± 1	10
GG-C	31 ± 1	72	82 ± 0	18	76 ± 1	10
GG-F	26 ± 3	72	89 ± 1	16	89 ± 1	12
M	10 ± 2	80	89 ± 1	11	91 ± 0	9
GJ	55 ± 7	66	94 ± 0	25	92 ± 0	9

GG-V, 'Green Gage'-Portugal from "Vila Viçosa" orchard; GG-C, 'Green Gage'-Portugal from "Cano" orchard; GG-F, 'Green Gage'-France; GJ, 'Golden Japan'; M, 'Mirabelle'. Mean ± standard deviation (n=10).

Quantification of Total Phenolic Compounds by Folin-Ciocalteu Method. The results obtained for the total phenolic compounds recovered from the flesh and the skin of all plum varieties are presented in **Figure 1**. Two standard curves, 5-caffeoylquinic acid (5CQA) and (-)-epicatechin (EC), were performed in this study since the quantification of total phenolics depends on the standard used.

The values obtained using the two standards show a higher estimation when 5CQA was used as compared to EC, for both flesh and skin. This is in agreement with the fact that 5CQA shows the lowest response factor, using the Folin–Ciocalteau reagent, compared to other phenolic standards (*23, 24*). In the present study, the ratio between the values of total phenolics calculated using EC and 5CQA as standards was not the same for all samples.

Plums of M and GG-F varieties showed a higher difference (83–86%) than the other plums (36–50%).

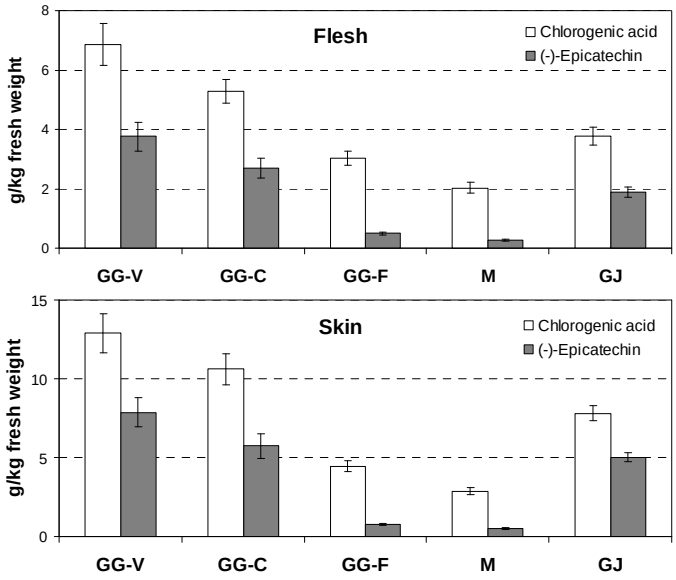

Figure 1. Total phenolic compounds of plums methanolic extracts by Folin-Ciocalteu method of the flesh and skin (mg/kg fresh weight), expressed as 5-caffeoylquinic acid and (-)-epicatechin (GG-C, 'Green Gage'-Portugal "Cano"; GG-F, 'Green Gage'-France; GG-V, 'Green Gage'-Portugal "Vila Viçosa"; GJ, 'Golden Japan'; M, 'Mirabelle').

The skin showed a significantly higher amount of phenolic compounds (1.5 to 2 fold) compared to the flesh tissue for all samples analyzed (**Figure 1**), which is in accordance with studies for other plum varieties (*13, 30*). The different varieties showed a wide variation on total phenolics content, for both flesh and skin. Total phenolic content ranged from 2.0 to 6.8 g 5CQA/kg and 0.3 to 3.8 g EC/kg of flesh. The phenolic content observed in this study is comparable to that previously reported with the same methodology and standard for other plums varieties (3.0 to 5.6 g 5CQA/kg) (*30).* Considering the results obtained on a 5CQA fresh weight basis: i) in the flesh, GG-V shows the highest amount of phenolic compounds, with about more 25% the amount found in GG-C and about 2 to 3 fold the amount present in GJ, GG-F, and M; ii) in the skin, GG-V and GG-C show the highest and a similar amount of phenolic compounds, with about 1.5 fold the amount of GJ and 2-3 fold the amount of GG-F and M. These results point out the much higher amount of phenolic compounds present on GG variety from Portugal from both orchards.

Quantification and Characterization of Phenolic Compounds by Reversed-Phase HPLC. RP-HPLC with photodiode array detection was used to separate, characterize, and quantify the different classes of phenolic compounds present in plums. An example of the RP-HPLC chromatograms obtained for skin before and after thiolysis, with the attribution of the main components to the peaks, is presented in **Figure 2**. For flesh tissues the chromatograms were similar except that there are no peaks corresponding to flavonols.

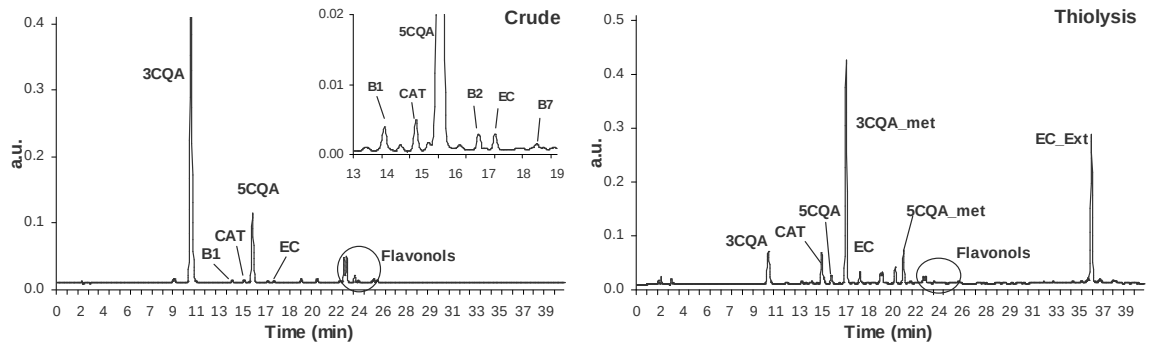

Figure 2. Reversed-Phase HPLC chromatograms (280 nm) of the crude extract and after thiolysis of plums (GG-C). (B1, procyanidin B1; B2, procyanidin B2; B7, procyanidin B7; CAT, (+)-catechin; 3CQA, 3-caffeoylquinic acid; 3CQA_met, 3-caffeoylquinic methyl ester; 5CQA, 5-caffeoylquinic acid; 5CQA_met, 5-caffeoylquinic methyl ester; EC, (-)-epicatechin; EC-Ext, (-)-epicatechin benzylthioether)

In the crude extract without thiolysis, compounds of the procyanidin type appeared as more or less unresolved peaks. After thiolytic depolymerization, two kinds of compounds are released: catechin or epicatechin terminal units depending on the nature of flavan-3-ol, and flavanol units linked to benzyl thioether corresponding to extension units, giving well resolved peaks which permitted accurate integration. As already mentioned for apple samples submitted to thiolysis (*20*), the thiolysis chromatograms showed additional peaks resulting from side reactions without significant consequences for quantification purposes: hydroxycinnamic acids are partly converted into their corresponding methylester and flavonols partly deglycosylated.

Phenolic compounds in the flesh. In the flesh of plum extracts, two classes of compounds, hydroxycinnamic acid derivatives and flavan-3-ols, were detected (**Table 2**). The main class of phenolic compounds quantified in the crude flesh extracts was the hydroxycinnamic acid derivatives. For GG variety the content of hydroxycinnamic acid derivatives ranged from 2.0 to 2.6 g/kg, which contrasts with the lower values for M (1.2 g/kg) and GJ (0.2 g/kg). In all varieties, 3-caffeoylquinic acid was the main phenolic compound, followed by 5-caffeoylquinic acid (**Table 2**). In GG flesh samples, 3-caffeoylquinic acid accounted for 81-83% of total hydroxycinnamic acids, whereas in M and GJ it represented slightly lower values (74 and 67%). These hydroxycinnamic acid derivatives have been also observed in previous studies on plums and in similar quantities (*2, 7, 13-15, 26, 30*). Although in small amounts, some other hydroxycinnamic acid derivatives were also detected by their characteristic UV spectra, which allowed their quantification by the 5-caffeoylquinic acid standard. A caffeic acid derivative, with a $[M-H]^-$ ion at *m*/*z* 335 and characteristic fragments at *m*/*z* 179, 161, and 135, was also detected. It was identified as 3-*O*-caffeoylshikimic acid by comparison with previously published MS data showing the same pseudomolecular ions and the same fragmentations for these compounds reported in dried plums (*31*). In addition, a 3-*O*-feruloylquinic acid was identified in M plums, with a MS spectrum that had a $[M-H]^-$ ion at *m*/*z* 367, in accordance with its molecular weight, and a fragment at *m*/*z* 193 that corresponded to the loss of the quinic acid moiety by MS fragmentation of the ester linkage (*31*). No evidences were obtained in the MS spectra for the presence of diCQA, neither for the corresponding *p*-coumaric and ferulic derivatives of shikimic acid.

Procyanidin B1 and (+)-catechin were the flavan-3-ols present in higher amount in all samples (**Table 2**). The flavan-3-ol monomers (-)-epicatechin and (+)-catechin were detected in the crude extracts for GG-C, GG-V, and GJ samples, being the (+)-catechin highly preponderant, while in M and GG-F samples only (+)-catechin was identified. Other studies of plums also describe (+)-catechin as the main monomeric flavan-3-ol detected, being (-)-epicatechin present in smaller amounts (*13-15*). Procyanidin B7 [dimer of (-)-epicatechin and (+)-catechin with a C4-C6 linkage] was identified only in the three samples of the GG variety. The procyanidin dimers of B-type were identified by retention time comparison in HPLC-photodiode array with the authentic markers available, and by their characteristic ESI-MS spectra (*m*/*z* 577, $[M-H]^-$; *m*/*z* 425, *retro*-Diels-Alder fragment;

and *m*/*z* 289 and 287, fragments of terminal and extension (+)-catechin residues in the dimer) (*14, 32, 33*). Other procyanidin dimers and trimers (characteristic fragments at *m*/*z* 865, 577, and 289) were also detected. Although they could not be fully identified, they were quantified as (+)-catechin equivalents using the corresponding calibration curve.

Table 2. Quantification of phenolic compounds in plum flesh (mg/kg of fresh weight and mg/fruit) by RP- HPLC, for crude extract and after thiolysis.

Crude Extract	**GG-V**		**GG-C**		**GG-F**		**M**		**GJ**	
	mg/kg	*mg/fruit*	*mg/kg*	*mg/fruit*	*mg/kg*	*mg/fruit*	*mg/kg*	*mg/fruit*	*mg/kg*	*mg/fruit*
Hydroxycinnamic acids										
3CQA (Rt 10.6 min)	1638 ± 50	46	1765 ± 58	39	2069 ± 74	39	879 ± 47	7	133 ± 4	5
5CQA (Rt 15.5 min)	162 ± 7	5	163 ± 7	4	165 ± 7	3	134 ± 5	1	27 ± 1	1
FQA* (Rt 15.0 min)	-	-	-	-	-	-	35 ± 3	1	-	-
CSA* (Rt 13.9 min)	65 ± 4	2	68 ± 3	2	97 ± 5	2	42 ± 2	1	20 ± 1	1
Unknown (Rt 9.3 min)	45 ± 2	1	37 ± 3	1	63 ± 7	1	47 ± 2	1	20 ± 3	1
Unknown (Rt 17.3 min)	-	-	-	-	60 ± 5	1	-	-	-	-
Unknown (Rt 19.3 min)	37 ± 2	1	35 ± 3	1	39 ± 1	1	51 ± 3	1	-	-
Unknown (Rt 20.5 min)	28 ± 3	1	30 ± 2	1	34 ± 1	1	-	-	-	-
Unknown (Rt 22.3 min)	30 ± 3	1	30 ± 2	1	43 ± 2	1	-	-	-	-
Subtotal	***2005***	***57***	***2128***	***49***	***2570***	***49***	***1188***	***12***	***200***	***8***
Flavan-3-ols										
B1 (Rt 13.4 min)	183 ± 14	5	140 ± 5	3	58 ± 3	1	54 ± 5	1	276 ± 10	10
CAT (Rt 14.8 min)	121 ± 3	3	119 ± 5	3	68 ± 3	1	15 ± 3	tr	220 ± 10	8
B2 (Rt 16.3 min)	109 ± 1	3	85 ± 4	2	36 ± 2	1	29 ± 4	tr	126 ± 4	5
EC (Rt 17.8 min)	79 ± 3	2	63 ± 2	1	-	-	-	-	61 ± 2	2
B7 (Rt 19.6 min)	22 ± 2	1	21 ± 1	1	26 ± 2	1	-	-	-	-
A (Rt 22.6 min)	-	-	-	-	-	-	-	-	78 ± 6	3
Unknown (Rt 14.3 min)	39 ± 4	1	34 ± 1	1	-	-	-	-	86 ± 3	3
Unknown (Rt 15.8 min)	55 ± 3	2	29 ± 1	1	-	-	-	-	64 ± 2	2
Unknown (Rt 16.8 min)	39 ± 2	1	40 ± 2	1	-	-	-	-	28 ± 1	1
Unknown (Rt 18.9 min)	42 ± 6	1	33 ± 1	1	-	-	-	-	33 ± 1	1
Unknown (Rt 20.2 min)	42 ± 6	1	36 ± 2	1	-	-	-	-	62 ± 6	2
Unknown (Rt 21.1 min)	50 ± 7	1	55 ± 3	1	54 ± 4	1	33 ± 3	tr	34 ± 7	1
Unknown (Rt 23.3 min)	-	-	-	-	-	-	38 ± 2	tr	65 ± 12	2
Subtotal	***781***	***21***	***655***	***16***	***242***	***5***	***169***	***1***	***1133***	***40***
TOTAL crude extract	**2786**	**78**	**2783**	**65**	**2812**	**54**	**1357**	**13**	**1333**	**48**
Thiolysis										
CAT	395 ± 10	11	497 ± 17	11	314 ± 14	6	194 ± 6	2	601 ± 28	22
EC	160 ± 4	4	198 ± 9	4	137 ± 8	3	123 ± 1	1	50 ± 2	2
A	-	-	-	-	-	-	-	-	242 ± 13	9
EC-Ext (Rt 36.0 min)	2640 ± 120	74	2743 ± 112	60	1674 ± 107	32	1402 ± 42	11	1568 ± 5	56
Unknown (Rt 23.9 min)	-	-	-	-	-	-	-	-	41 ± 13	1
Subtotal	***3195***	***89***	***3438***	***75***	***2125***	***41***	***1719***	***14***	***2502***	***90***
TOTAL	**5200**	**146**	**5566**	**124**	**4695**	**90**	**2907**	**26**	**2702**	**98**
DPn	**8.5 ± 0.1**		**6.6 ± 0.1**		**5.4 ± 0.1**		**5.7 ± 0.2**		**4.5 ± 0.2**	

Flavan-3-ols are expressed as catechin equiv and hydroxycinnamic acids as 5-caffeoylquinic acid equiv. A, A-type procyanidin, B1, procyanidin B1; B2, procyanidin B2; B7, procyanidin B7; CAT, (+)-catechin; 3CQA, 3-caffeoylquinic acid; 5CQA, 5-caffeoylquinic acid; CSA, caffeoylshikimic acid; DPn, average degree of polymerization; EC, (-)-epicatechin; EC-Ext, (-)-epicatechin benzylthioether; FQA, feruloylquinic acid; GG-C, 'Green Gage'-Portugal "Cano"; GG-F, 'Green Gage'-France; GG-V, 'Green Gage'-Portugal "Vila Viçosa"; GJ, 'Golden Japan'; M, 'Mirabelle'; Rt, Retention time, tr, trace amounts, <0.5 mg/fruit. Mean ± standard deviation (n=3). *Identification only by ESI-MS, no standard available.

While the main class of phenolic compounds quantified in the crude extract was the hydroxycinnamic acid derivatives for GG (72-91% of total phenolics quantified in crude extract) and M (88%) varieties, GJ showed a very different phenolic composition. For this plum, the class of flavan-3-ols was the main class of phenolic compounds (85% of total), B1 was the major procyanidin identified, and an A-type procyanidin dimer was only identified in this plum. The A-type procyanidin dimer detected (characteristic MS fragments at *m*/*z* 575, $[M-H]^-$; *m*/*z* 423, *retro*-Diels-Alder fragment; and *m*/*z* 287 and 285)

showed an ESI-MS spectra similar to those of the B-type dimers, but with two mass units less, as expected for this type of molecule with two inter-catechin bonds. Procyanidin dimers (B1, B2, and A-type dimers) have already been described as the main phenolic compounds in the flesh of other plums, although in smaller amounts than observed for GJ plums (*13*). This work also confirms the occurrence of B7 dimers previously reported to be present in plums (*34*).

Since the thiolysis reaction causes depolymerization, it gives access to the RP-HPLC quantification of the part of procyanidins which are not accessible to quantification when the crude extract was analyzed. As a consequence, on the basis of RP-HPLC analysis of the thiolysed samples (**Table 2**), the amount of phenolic compounds quantified was higher than in crude extracts for all varieties (about 2 fold), indicating the presence of high concentrations of procyanidins. As observed for the crude extract, plums of the 'Green Gage' variety (GG-V, GG-C, and GG-F) presented the highest phenolic content, when compared to M and GJ. The amount of phenolics of these plums after thiolysis ranged from 5.7 g/kg of fresh weight for GG-C to 2.7 g/kg for GJ, which shows that some of these varieties, namely GG, are very rich in phenolic compounds. On a fruit basis, the amount of phenolic compounds ranged from 146 and 124 mg/fruit for GG-V and GG-C, respectively, to 26 mg/fruit for M. On a fruit basis, the Portuguese 'Green Gage' plums and GJ plums, the biggest one, were the richest when compared to M variety and to GG-F (**Table 2**). The average quantity of phenolic compounds calculated after RP-HPLC analysis in other studies for the plums flesh was usually lower, 1.1 g/kg of fresh weight (*1, 12, 14*), 2.8 g/kg (*13*), 2.7 g/kg (*26*), and 3.7 g/kg (*15*), probably because data here presented take into account the amount of polymerized procyanidins.

As expected, after thiolysis, an increase of total flavanols (i.e. the sum of (+)-catechin, (-)-epicatechin, and (-)-epicatechin benzylthioether) was observed. As a consequence, the class of flavan-3-ols became the main class of phenolic compounds identified for all samples analyzed, 92% in GJ, 62% in GG-C, 61% in GG-V, and 54% in M, except for GG-F (45%). These results may explain the differences in the ratio between the values of total phenolics calculated using EC and 5CQA as standards observed by the Folin-Ciocalteu method, suggesting that the higher ratio EC/5CQA is related to a higher proportion of hydroxycinnamic acids in relation to flavan-3-ols in samples.

Terminal units of procyanidins observed in plums are mainly (+)-catechin (54-77% of all terminal units). In GJ, as previously observed in the pericarp of lichi fruits (*28*), A-type procyanidin was detected after thiolysis in higher quantity than in crude extract, which suggests its presence as native dimers and also as terminal residues in the structure of procyanidin oligomers.

RP- HPLC analyses following thiolysis allows the determination of the average degree of polymerization (DPn) of procyanidins (**Table 2**, bottom line). The flesh of GG-V plums showed the highest polymerized procyanidins, with a DPn of approximately 8.5, followed by GG-C (DPn=6.6). GG-F and M showed a DPn of 5.4 and 5.7, respectively, and the lowest DPn of flesh procyanidins was observed for GJ (DPn=4.5).

*Phenolic compounds in the skin***.** The total content of phenolic compounds on a weight basis was much higher in the skin than in the flesh, in agreement with other studies in plums (*6, 14, 30*). However, the contribution of the phenolic compounds of the skin to the overall content of phenolic compounds of the fruit is lower than that of the flesh (**Tables 2** and **3**). This is due to the lower proportion of skin weight when compared to the flesh of the fruit (Table 1), which ranged from 14, 22-25, and 38%, respectively for M, the three GG, and GJ plums.

The main hydroxycinnamic acid derivatives and flavan-3-ols detected and quantified in the flesh were also identified in the skin of the plums, as is shown in **Table 3**. As observed in the flesh, the main class of phenolic compounds quantified in the crude extract of the skin was the hydroxycinnamic acid derivatives ranging from 0.6 to 4.7 g/kg, except for GJ. For the latter variety, the main class of phenolic compounds was the flavan-3-ols (81% of total phenolic compounds), similarly to what was found for the flesh. Also, 3-caffeoylquinic acid was, by far, the most abundant hydroxycinnamic acid derivative and GG variety were those that accounted for the higher percentage of this compound (75-77% of total hydroxycinnamic acids). Procyanidin B1 and (+)-catechin were the flavan-3-ols present in higher amount in all samples, in about similar proportions. As observed for the flesh, procyanidin B7 was found in GG-V and GG-C samples, although not in GG-F, and A-type procyanidin was only found in GJ.

Table 3. Quantification of phenolic compounds in plum skin (mg/kg of fresh weight and mg/fruit) by RP-HPLC, for crude extract and after thiolysis.

Crude Extract	GG-V		GG-C		GG-F		M		GJ	
	mg/kg	*mg/fruit*	*mg/kg*	*mg/fruit*	*mg/kg*	*mg/fruit*	*mg/kg*	*mg/fruit*	*mg/kg*	*mg/fruit*
Hydroxycinnamic acids										
3CQA (Rt 10.6 min)	3254 ± 15	20	3241 ± 43	19	3650 ± 175	15	1343 ± 63	1	435 ± 14	6
5CQA (Rt 15.5 min)	722 ± 19	4	603 ± 9	4	544 ± 5	2	869 ± 18	1	77 ± 5	1
FQA* (Rt 15.0 min)	-	-	-	-	-	-	40 ± 2		-	-
CSA* (Rt 13.9 min)	137 ± 6	1	130 ± 5	1	218 ± 2	1	74 ± 2	tr	69 ± 2	1
Unknown (Rt 9.3 min)	87 ± 3	1	81 ± 5	1	133 ± 1	1	66 ± 3	tr	31 ± 1	1
Unknown (Rt 17.3 min)	-	-	-	-	111 ± 4	1	44 ± 2	tr	-	-
Unknown (Rt 19.3 min)	66 ± 2	1	50 ± 4	tr	-	-	123 ± 8	tr	-	-
Unknown (Rt 20.5 min)	44 ± 0	tr	47 ± 4	tr	-	-	-	-	-	-
Unknown (Rt 22.3 min)	42 ± 10	tr	44 ± 4	tr	63 ± 0	tr	42 ± 1	tr	-	-
Subtotal	***4352***	***27***	***4196***	***25***	***4719***	***20***	***2601***	***3***	***612***	***9***
Flavan-3-ols										
B1 (Rt 13.4 min)	247 ± 6	1	232 ± 5	1	117 ± 4	1	164 ± 15	tr	879 ± 10	12
CAT (Rt 14.8 min)	178 ± 5	1	184 ± 2	1	114 ± 1	1	117 ± 5	tr	1044 ± 26	15
B2 (Rt 16.3 min)	125 ± 2	1	114 ± 3	1	69 ± 2	tr	-	-	279 ± 8	4
EC (Rt 17.8 min)	86 ± 5	1	84 ± 2	1	95 ± 6	1	-	-	119 ± 4	2
B7 (Rt 19.6 min)	31 ± 1	tr	28 ± 1	tr	-	-	-	-	-	-
A (Rt 22.6 min)	-	-	-	-	-	-	-	-	342 ± 14	5
Unknown (Rt 12.7 min)	28 ± 1	tr	29 ± 1	tr	-	-	107 ± 8	tr	110 ± 7	2
Unknown (Rt 15.8 min)	50 ± 6	tr	46 ± 1	tr	-	-	-	-	326 ± 8	5
Unknown (Rt 16.8 min)	43 ± 3	tr	43 ± 1	tr	-	-	-	-	56 ± 8	1
Unknown (Rt 18.9 min)	45 ± 4	tr	44 ± 2	tr	-	-	-	-	19 ± 1	tr
Unknown (Rt 20.2 min)	52 ± 6	tr	51 ± 2	tr	-	-	-	-	259 ± 15	4
Unknown (Rt 21.1 min)	53 ± 2	tr	55 ± 2	tr	63 ± 1	tr	54 ± 1	tr	22 ± 1	tr
Unknown (Rt 23.3 min)	96 ± 1	1	83 ± 9	1	-	-	-	-	140 ± 12	2
Subtotal	***1034***	***6***	***993***	***6***	***458***	***2***	***442***	***1***	***3595***	***52***
Flavonols										
Myr (Rt 22.7 min)	154 ± 5	1	110 ± 2	1	146 ± 41	1	268 ± 30	tr	-	-
Rut (Rt 22.9 min)	188 ± 2	1	110 ± 3	1	160 ± 1	1	436 ± 12	1	-	-
Hyper (Rt 23.2 min)	-	-	-	-	-	-	-	-	29 ± 4	tr
Isoquer (Rt 23.5 min)	49 ± 1	tr	43 ± 3	tr	40 ± 5	tr	133 ± 3	tr	27 ± 2	tr
Isorham-rut (Rt 25.3 min)	35 ± 2	tr	23 ± 2	tr	42± 1	tr	-	-	-	-
Quer (Rt 25.9 min)	-	-	-	-	-	-	-	-	52 ± 5	1
Unknown (Rt 19.3 min)	-	-	-	-	-	-	-	-	34 ± 3	1
Unknown (Rt 23.8 min)	25 ± 1	tr	20 ± 2	tr	31 ± 2	tr	62 ± 4	tr	53 ± 4	1
Unknown (Rt 25.0 min)	42 ± 1	tr	30 ± 2	tr	56 ± 5	tr	39 ± 1	tr	27 ± 2	tr
Subtotal	***493***	***3***	***336***	***2***	***475***	***2***	***938***	***1***	***222***	***3***
TOTAL crude extract	**5879**	**36**	**5525**	**33**	**5652**	**24**	**3981**	**5**	**4429**	**64**
Thiolysis										
CAT	636 ± 29	4	765 ± 8	5	540 ± 7	2	284 ± 13	tr	1444 ± 38	20
EC	231 ± 12	1	234 ± 7	1	198 ± 15	1	127 ± 1	tr	103 ± 4	1
A	-	-	-	-	-	-	-	-	605 ± 20	8
EC-Ext (Rt 36.0 min)	2904 ± 128	17	3204 ± 66	19	2155 ± 0	9	878 ± 76	1	2316 ± 21	32
Unknown (Rt 23.9 min)	69 ± 2	23	-	-	101 ± 5	1	-	-	98 ± 5	1
Subtotal	***3840***	***45***	***4203***	***25***	***2994***	***13***	***1289***	***1***	***4566***	***62***
TOTAL	**8685**	**75**	**8735**	**52**	**8188**	**35**	**4828**	**5**	**5400**	**74**
DPn	**5.8 ± 0.1**		**5.6 ± 0.1**		**5.1 ± 0.4**		**4.0 ± 0.1**		**5.5 ± 0.2**	

Flavan-3-ols are expressed as catechin equiv, hydroxycinnamic acids as 5-caffeoylquinic acid equiv, and flavonols as rutin equiv. Flavan-3-ols are expressed as catechin equiv and hydroxycinnamic acids as 5-caffeoylquinic acid equiv. A, A-type procyanidin; B1, procyanidin B1; B2, procyanidin B2; B7, procyanidin B7; CAT, (+)-catechin; 3CQA, 3-caffeoylquinic acid; 5CQA, 5-caffeoylquinic acid;CSA, caffeoylshikimic acid; DPn, average degree of polymerization; EC, (-)-epicatechin; EC-Ext, (-)-epicatechin benzylthioether; FQA, feruloylquinic acid; GG-C, 'Green Gage'-Portugal "Cano"; GG-F, 'Green Gage'-France; GG-V, 'Green Gage'-Portugal "Vila Viçosa"; GJ, 'Golden Japan'; Hyper, Hyperoside; Isoquer, isoquercitrin; Isorham-rut, isorhamnetin rutinoside; M, 'Mirabelle'; Myr, myricetin; Quer, Quercitrin; Rt, retention time; Rut, rutin; tr, trace amounts, <0.5 mg/fruit. Mean ± standard deviation (n=3).*Identification only by ESI-MS, no standard available.

In the skin, another class of phenolic compounds, the flavonols, was detected. For crude methanol extract, they accounted for 8% or less of the total phenolics, except for the M variety, for which they represented 24% (**Table 3**). Similar relative amounts of flavonols, as those occurring in the M variety, have been also reported for other plums varieties (eg. 'Autumn Sweet', 'Beltsville Elite', 'B70197', 'Castleton', 'Empress', 'Longjohn', and 'Stanley') (*26*). The main flavonol compound was rutin (rhamnoglucoside of quercetin), which accounted for 0.4 g/kg of skin in M and 0.1-0.2 g/kg of skin in GG

varieties, followed by myricetin, although these two flavonols have not been identified in GJ. In addition, quercitrin and hyperoside were only identified in GJ plums. Previous studies on plums also showed rutin as the principal flavonol, ranging from 10 to 100 mg/kg (*7, 13, 14, 26, 30*). Various quercetin glycosides, as monosaccharides or disaccharides, were previously found in plums, like quercetin glucoside (isoquercitrin), quercetin rhamnoside (quercitrin), and quercetin xyloside, and in smaller amounts kaempferol 3-rutinoside, 3-glucoside, 3-galactoside, and 3-arabinoside-7-rhamnoside (*1, 12, 13*). The total amount of flavonols that have been reported to be present in plums skin ranged from 30 to 350 mg/kg of fresh weight, depending on the variety (*12, 13, 26*). The occurrence of these flavonols in higher quantities in the varieties under study should be exploited, as some of these compounds, such as quercetin glycosides, show relevant biological activities (*19*).

After thiolysis, the total amount of phenolic compounds increased, but to a lesser extent compared to the flesh, being noticeable only for the three GG samples and, among these, more substantially for GG-V and GG-C. This indicates a possible presence of lower proportions of procyanidins in the skin, when compared to the flesh. Flavan-3-ols accounted for 85% of total phenolic compounds in the skin of GJ, 48% in GG-C, 44% in GG-V, 37% in GG-F, and 27% in M. The total amount of phenolic compounds quantified by RP-HPLC in skin ranged from 8.7 g/kg of fresh weight for GG-C and GG-V to 4.8 g/kg for M. As it was observed for the flesh, the Portuguese ‘Green Gage’ plums were richer (p<0.05) in phenolic compounds when compared to GG-F (8.2 g/kg) and to the other plums analyzed. The A-type procyanidin of GJ increased about 2 fold with thiolysis, which means that this dimer is present in GJ skin and flesh as native and as terminal unit of the procyanidin oligomers.

As observed in the flesh, the GG-V and GG-C plums presented the highest polymerized procyanidins in the skin, with a DPn of 5.8 and 5.6, respectively, which however were lower than that of the flesh (**Table 3**, bottom line). The procyanidins of GG-F skin had similar DPn (5.1) of those present in the flesh, and the procyanidins of GJ showed a slightly higher DPn than those of the flesh (DPn=5.5).

Principal Component Analysis (PCA). A comparative analysis of the phenolic compounds present in five samples studied was performed by means of PCA both in the flesh (**Figure 3**) and in the skin (**Figure 4**) as an attempt to identify the different plums based on their phenolic compounds contents.

Figure 3a shows the scores scatter plot (PC1 x PC2) of the data provided by the analysis of phenolic compounds of the flesh, where four groups were obtained. This analysis allows the differentiation of the Portuguese GG samples (PC1 positive and PC2 negative), GG-F (PC2 positive), M (PC1 negative and PC2 positive), and GJ (PC1 and PC2 negative). These two axes contain 99% of the total variability. According to the loadings plot (**Figure 3b,c**), the distinction of the GG samples is due to the higher content of hydroxycinnamic acids, mainly 3-caffeoylquinic acid, and the flavan-3-ols, mostly the procyanidins. GG-C and GG-V are distinguished from GG-F due to the higher content of procyanidins in Portuguese GG samples. GJ plum is distinguished from the other varieties due to the presence of A-type procyanidin, procyanidin B1, and (+)-catechin.

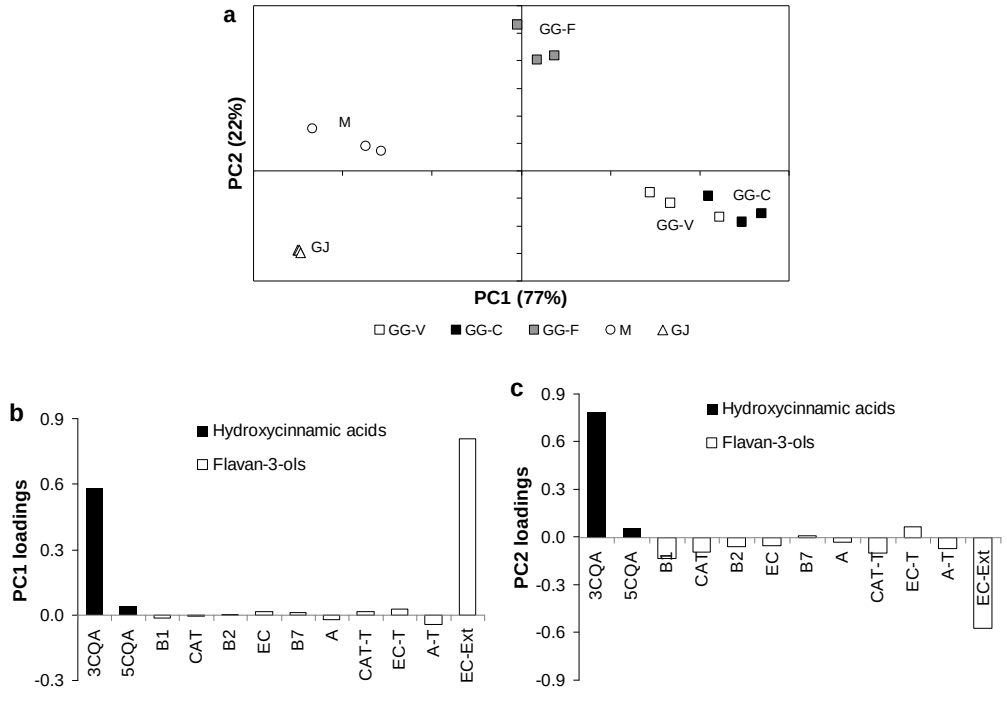

Figure 3. PCA of the phenolic compounds of plums flesh: (a) scores scatter plot (PC1 vs. PC2); and (b) PC1 loadings (c) PC2 loadings. A, A-type dimeric procyanidin; A-T, A-type terminal dimeric procyanidin; B1, procyanidin B1; B2, procyanidin B2; B7, procyanidin B7; CAT, monomeric (+)-catechin; CAT-T, terminal (+)-catechin; 3CQA, 3-caffeoylquinic acid; 5CQA, 5-caffeoylquinic acid; EC, monomeric (-)-epicatechin; EC-Ext, extension (-)-epicatechin; EC-T, terminal (-)-epicatechin.

The PC1 x PC2 scores scatter plot of phenolic compounds present in skin distinguished the GG varieties (PC1 and PC2 positive) from M (PC1 positive and PC2 negative) and GJ (PC1 and PC2 negative) (results not shown). The loadings plots (PC1 results not shown) demonstrated that GG plums were separated according to their content in 3-caffeoylquinic acid, procyanidin B7, (-)-epicatechin terminally-linked to procyanidins, and the flavonol isorhamnetin rutinoside. The PC2 x PC3 scores scatter plot (**Figure 4a**) distinguished the Portuguese GG variety (PC2 positive and PC3 negative) from GG-F (PC2 and PC3 positive), and M and GJ (PC2 and PC3 negative). According to the loadings plot (**Figure 4b,c**), the distinction of the GG samples from the others is due to the higher content of procyanidins, namely, the oligomeric (-)-epicatechin and (+)-catechin, and the dimers B2 and B7. GG-C and GG-V are distinguished from GG-F due to the presence of procyanidin B7 in GG-V and GG-C and not in GG-F skin, and the higher content of oligomeric procyanidins.

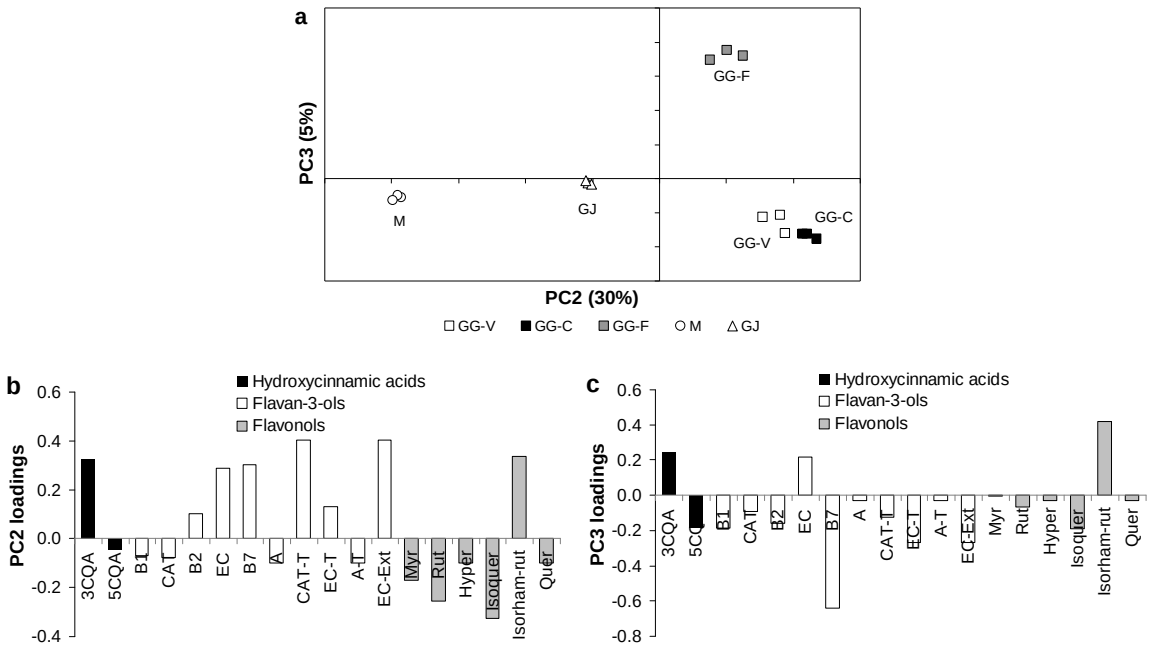

Figure 4. PCA of the phenolic compounds of plums skin: (a) scores scatter plot (PC2 vs. PC3); and (b) PC2 loadings (c) PC3 loadings. A, A-type dimeric procyanidin; A-T, A-type terminal dimeric procyanidin; B1, procyanidin B1; B2, procyanidin B2; B7, procyanidin B7; CAT, monomeric (+)-catechin; CAT-T, terminal (+)-catechin; 3CQA, 3-caffeoylquinic acid; 5CQA, 5-caffeoylquinic acid; EC, monomeric (-)-epicatechin; EC-Ext, extension (-)-epicatechin; EC-T, terminal (-)-epicatechin; Hyper, Hyperoside; Isoquer, isoquercitrin; Isorham-rut, isorhamnetin rutinoside; Myr, myricetin; Quer, Quercitrin; Rut, rutin.

The data obtained by thiolysis followed by RP-HPLC and chemometrics of the plums phenolic compounds allowed to observe that the plums from different variety and specie and even plums of the same variety but from different origins could be distinguished according to their characteristic phenolic composition of the flesh and/or the skin. These diagnostic compounds identified here should however be confirmed using for comparison other varieties, harvests, and stages of ripening. Anyway, they emphasize the importance of the geographical local of production (resulting in possible different production procedures and climate influences) for the total phenolics content of plums.

ABBREVIATIONS USED

A, procyanidin A; A-T, procyanidin A terminal dimmer; B1, procyanidin B1; B2, procyanidin B2; B7, procyanidin B7; CAT, (+)-catechin; CAT-T, (+)-catechin terminal units; 3CQA, 3-caffeoylquinic acid; 3CQA_met, 3-caffeoylquinic methyl ester; 5CQA, 5-caffeoylquinic acid; 5CQA_met, 5-caffeoylquinic methyl ester; CSA, caffeoylshikimic acid; DPn, average degree of polymerization; EC, (-)-epicatechin; EC-Ext, (-)-epicatechin benzylthioether; EC-T, (-)-epicatechin terminal units; ESI-MS, electrospray ionization - mass spectrometry; FQA, feruloylquinic acid; GG-C, 'Green Gage'-Portugal "Cano"; GG-F, 'Green Gage'-France; GG-V, 'Green Gage'-Portugal "Vila Viçosa"; GJ, 'Golden Japan'; Hyper, Hyperoside; Isoquer, isoquercitrin; Isorhamnetin, isorhamnetin rutinoside; M, 'Mirabelle'; Myr, myricetin; Quer, Quercitrin; RP-HPLC, reversed-phase - high-performance liquid chromatography; Rut, rutin.

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Study of the volatile components of a candied plum and estimation of their contribution to the aroma

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Abstract

A methodology comprising the combination of sample analysis by simultaneous distillation and extraction (SDE) and headspace analysis by solid phase microextraction (HS-SPME) is proposed to characterize the volatile fraction and to estimate its contribution to the aroma properties of heat treated sugar-rich fruits. In the present study, the volatile composition of "Ameixa d'Elvas", a traditional candied plum, was established by SDE. It reflects the complexity of the reactions and rearrangements that happen during the fruit ripening plus those occurring due to the thermal processing in presence of sucrose. HS-SPME allowed to detect the volatile compounds present in the headspace of the intact pulp, potentially responsible for the aroma properties. Eleven compounds were determined as the would-be impact odourants of "Ameixa d'Elvas", which are associated with sweet, cooked, and fruity odours. All would-be impact odourants of "Ameixa d'Elvas" fruits, except one, were also detected in the sucrose syrup headspace where the fruits have been submersed. This study indicates that, together with the pulp of the processed fruit, syrup contains compounds that can contribute to the aroma of candied fruits.

Keywords: Ameixa d'Elvas; *Prunus domestica*; headspace analysis; volatile compounds; syrup; would-be impact odourants

1. Introduction

"Ameixa d'Elvas" is a "Protected Designation of Origin" recognized by the European Union for a processed plum from Elvas (south-east of Portugal) obtained by a traditional candying process. Only the fruits of a special type of Green Gage plum, 'Rainha Cláudia Verde' (*Prunus domestica* L.) variety, can be utilized to produce "Ameixa d'Elvas". The candying process consists in boiling the intact plums in water for 15 min and then putting them in sucrose syrup, which is successively concentrated until 75 ºBrix. The plums can be consumed with this syrup or, alternatively, can be stored in the syrup until being washed and packed in a solid state. Several chemical reactions occur during "Ameixa d'Elvas" processing which composition was never objectively described (Diário da República, 1994) neither identified.

Analysis of the volatile composition of fresh plums showed that the esters are qualitatively the most important class of compounds (Crouzet, Etievant & Bayonove, 1990). Quantitatively, they are also the major components of the volatile composition of fresh plums, followed by alcohols or aldehydes, depending on the cultivar and on the methodology used for volatile extraction. The contribution of these components for the overall aroma has been evaluated calculating their sensory perception limit (SPL) values in model matrices. Nonanal, 1-hexanol, *cis*-3-hexenol, linalool, benzaldehyde, γ-octalactone, and γ-decalactone were considered as important contributors to the aroma of fresh plums (Crouzet et al., 1990; Williams & Ismail, 1981).

The aroma of fruits can change on heating due to the liberation of volatile compounds from glycosidic precursors, oxidation, water addition, and cyclization of individual compounds, such as hydroxy acids (Belitz, Grosch & Schieberle, 2004). Thermal processes applied to plums induce modifications of their volatile composition, which have been described in literature for several types of thermal processes in the presence of high quantities of sucrose (Crouzet et al., 1990; Ismail, Williams & Tucknott, 1980b). The thermal processing is known to induce chemical modifications of the product components, mainly sugars and amino acids, leading to the production of a wide spectrum

of flavour compounds. Carbonyl compounds are important for the aroma in heated processed plums, being benzaldehyde, furfural, 2-furfurylmethylketone, and nonanal the major constituents of the headspace and giving rise to almond-like and woody sensory descriptors (Ismail et al., 1980b). The molecules present in the headspace are indeed responsible for the smell that is perceived by the olfactory system if they are in concentrations above their SPL.

An approach to estimate the contribution of the volatile compounds to the aroma is the calculation of the aroma index (*I*) for each compound and the identification of the would-be impact odourants (Rocha, Rodrigues, Coutinho, Delgadillo & Coimbra, 2004). This was done relating the estimated concentration of each volatile component by simultaneous distillation and extraction (SDE), followed by gas chromatography-quadrupole mass spectrometry (GC-qMS) analysis, with the corresponding SPL value reported in literature. This methodology, which does not include the use of sensory panel and/or GC-olfactometry studies, has been shown to be suitable as a preliminary step for estimating the potential contribution of the volatile components to the aroma properties. However, on the other hand, rearrangements, hydrolysis or artefact compound generation during water distillation, due to decomposition of the matrix components and impurities that may be introduced from solvents, have been extensively reported (Boulanger & Crouzet, 2000; Chaintreau, 2001; Kataoka, Lord & Pawliszyn, 2000). As SDE promotes an exhaustive extraction and provokes the dispersion of the solid sample in the liquid phase, it is possible that not all the compounds recovered by SDE are emitted by the intact sample and/or occur in their headspace.

Solid phase microextraction (SPME) is a rapid, easy, solvent-free and sensitive sampling technique, evidenced by studies from a large number of fruits and products (Kataoka et al., 2000). The methodology that comprises SPME associated with GC-qMS is able to identify and quantify the volatile compounds, namely the compounds that occur in the headspace of different matrices. An advantage of SPME over the conventional solvent extraction methods is that the extracts do not have to be concentrated prior to analysis, preventing losses of low boiling point volatiles, such as short chain acids, alcohols and aldehydes (Chaintreau, 2001; Rocha, Ramalheira, Barros, Delgadillo & Coimbra, 2001), and could allow their detection, which is usually impossible to do due to their co-elution with the solvent. Using SPME it is also possible to analyse the volatile composition present

in the headspace of fruits, which is potentially responsible for their aromas. SPME technique also allows to evaluate the formation of compounds during SDE due to oxidation and/or thermal reactions.

The aim of this work is to study the volatile composition of a traditional product, "Ameixa d'Elvas" plums, and estimate the contribution of the volatile compounds to the aroma, identifying the would-be impact odourants present in the plums headspace. The volatile composition was determined using SDE followed by GC-qMS analysis and calculating the aroma index for each compound. HS-SPME coupled to GC-qMS was used to evaluate which of the volatile compounds identified as having $I>1$ were in fact present in the headspace of "Ameixa d'Elvas" (would-be impact odourants). Furthermore, the headspace volatile profile of the 75 ºBrix sucrose syrup used to process and store "Ameixa d'Elvas" was also studied by HS-SPME-GC-qMS.

2. Materials and methods

2.1. Samples

Plums of "Rainha Cláudia Verde" (*Prunus domestica* L.) variety in a precisely established stage of ripening (16 – 17 ºBrix, pH 3.3, and titratable acidity of 1 meq of malic acid per 100 g of plum) were boiled in water for 15 min. The intact boiled plums were than immersed in 60 ºBrix sucrose syrup. The solution was concentrated to 65 ºBrix in the following day and to 75 ºBrix after 7 days. The plums were stored, in storage tanks containing 400 Kg of fruits, two months in the 75 ºBrix sucrose syrup which concentration was occasionally (2 or 3 times) corrected due to its hygroscopicity. The processed plums (10 Kg), "Ameixa d'Elvas", were collected in the factory, Confibor Lda, Estremoz, Portugal, brought to the laboratory and maintained in their 75 ºBrix syrup until analyses.

2.2. Materials

Dichloromethane used was of analytical grade and was purchased from Sigma-Aldrich Inc. (Bellefonte, PA, USA). A SPME holder was used to perform headspace SPME manually. SPME holder for manual sampling and fibre used in the analyses were

purchased from Supelco Inc. (Bellefonte, PA, USA). SPME device included a fused silica fibre, partially cross-linked with 65 μm Carbowax-divinylbenzene (CW/DVB), which was conditioned according to the manufacturer's recommendations (250 ºC for 30 min in the GC injector). The CW/DVB coating fibre was selected because it is a mixed coating that contains a liquid polymer and solid particles (Pillonel, Bossett & Tabacchi, 2002). This type of coating combines the absorption properties of the liquid polymer with the adsorption properties of porous particles, which contains macro (>500 Å), meso (20-500 Å) and microporous (2-20 Å). The mutually synergetic effect of adsorption and absorption to the stationary phase promotes a high retention capacity and, consequently, a higher sensitivity than fibres based on absorption only. The CW/DVB coating fibre is recommended for smaller and polar molecules (molecular weight between 40 and 275) and seems to be adequate for the analysis of complex matrix (Roberts, Pollien & Milo, 2000; Song, Fan & Beaudry, 1998), such as "Ameixa d'Elvas" plums.

2.3. SDE analysis

The "Ameixa d'Elvas" plums (± 250 g, corresponding to 10 processed fruits), after removal of the stones, were immersed in distilled water (600 mL), and 100 μL of pentanoic acid (2 μL/mL) was added as internal standard. The samples were submitted to the volatile extraction in a modified Likens-Nickerson apparatus (Rocha, Delgadillo & Correia, 1996; Schultz, Flath, Mon, Eggling & Teranishi, 1977) for 3 h using bi-distilled dichloromethane (70 mL). After few minutes of boiling extraction, an aqueous slurry of pulp was formed. The dichloromethane extracts were cooled to -20 ºC to separate the frozen water from the organic phase by decantation and then the extracts were dried over anhydrous sodium sulphate. Each extract was concentrated to about 1 mL by distillation using a Vigreux column under low pressure at room temperature and a trap with liquid nitrogen on top. The concentrated extracts were stored in a glass screw-top vial at -20 ºC until being analyzed by GC-qMS. Three independent extractions were done and each extract was injected twice into the GC-qMS.

2.4. Headspace-SPME analysis

The "Ameixa d'Elvas" plums (60±5 g, depending on their size, corresponding to 2-3 processed fruits), open in two halves, were placed into a 130 mL glass vial, which corresponds to a ratio of the volume of the solid phase to the headspace volume (1/β) of 0.5. The sucrose syrup (63±3 g) was placed into a 130 mL glass vial, 8 g of NaCl was added to promote the salting out of the volatile compounds (results not shown), and the solution was stirred using a magnetic bar. The 1/β ratio was 0.5. The vials containing both types of samples (plum and syrup) were capped with a cap containing a butyl-rubber septum (Sigma-Aldrich Inc., Bellefonte, PA, USA) and placed in a thermostatted bath adjusted to 40.0±0.1 ºC to promote the transference of the compounds from the sample to the headspace. Two different times (60 and 120 min) of partition between the sample and headspace were tested. Partition time of 60 min between the sample and headspace was chosen for the analysis, because no significant differences were observed in the chromatographic areas with increasing the time to 120 min (results not shown). After this step, the SPME fibre was manually inserted into the sample vial headspace and the compounds were extracted for 45 min.

Blanks, corresponding to the analysis of the coating fibre not submitted to any extraction procedure, were run between sets of three analyses. All measurements were made with, at least, five replicates, being each replicate the analysis of one different aliquot (plum or syrup).

2.5. GC-qMS analysis

The dichloromethane extracts from SDE and the desorbed volatile compounds from SPME were separated and analyzed on a GC-qMS, Agilent Technologies 6890 N Network gas chromatograph. The GC was equipped with a 30 m x 0.32 mm (i.d.) DB-FFAP fused silica capillary column (J&W Scientific, Folsom, CA, USA), 0.25 μm film thickness, connected to an Agilent 5973 mass selective detector. Injections were made in splitless mode (5 min) and the injector was at 250 ºC.

Some specific chromatographic conditions were used:

i) For SDE, the volatile extract (1 μL) was injected in the injection port lined with a 4.0 mm i.d. splitless glass liner. The detector started to operate after 8 min of injection (solvent delay).

ii) For SPME, the injection port was lined with a 0.75 mm i.d. splitless glass liner. The fibre, containing the headspace volatile compounds, was introduced into the injector for 5 min for desorption of the compounds. The detector started to operate immediately after the injection (no solvent delay).

The oven temperature was programmed from 35 to 220 ºC at 2 ºC/min rate and the transfer line was heated at 250 ºC. The helium carrier gas had a column head pressure of 12 psi. The mass spectrometer was operated in the electron impact mode at 70 eV, scanning the range *m/z* 30-300 in a 1 s cycle, in a full scan mode acquisition. Identification of volatile compounds was achieved by comparison of the GC retention times and mass spectra with those, when available, of the pure standard compounds. All mass spectra were also compared with the library data system of the GC-qMS equipment (Wiley 275).

Estimated concentrations for all compounds were made by GC peak area comparisons of the SDE extract components with the area of a known quantity of internal standard (pentanoic acid). The GC peak area data obtained by the HS-SPME analysis were used as an indirect approach to estimate the relative content of each volatile compound. The reproducibility of the results was expressed as standard deviation in tables.

3. Results and discussion

3.1. Identification and quantification of "Ameixa d'Elvas" volatile composition

A total ion chromatogram of the SDE analysis of "Ameixa d'Elvas" plum is shown in Fig. 1a, with the attribution of peak numbers of the main components (shown in Table 1). This methodology allowed to identify and quantify seventy compounds, representing a total concentration of 49.4 mg/kg of pulp (Table 1). The acids, accounting for 22.6 mg/kg of pulp, represented the major group of compounds with 46% of the total concentration. From the 12 acids identified, hexadecanoic acid was the most abundant (16.5 mg/kg of pulp). Almost all other saturated even numbered straight chain fatty acids were also

present: C_{14} (1.5 mg/kg), C_{12} (2.4 mg/kg), C_{10} (0.8 mg/kg), C_8 (0.3 mg/kg), C_6 (0.1 mg/kg), and C_2 (0.1 mg/kg), together with the odd numbered straight chain fatty acids C_{15} (0.5 mg/kg) and C_9 (0.2 mg/kg), the branched 3-methyl butanoic acid (0.2 mg/kg) and 2-ethyl hexanoic acid (0.01 mg/kg), and benzoic acid (0.02 mg/kg of pulp). From these, only hexadecanoic acid and 3-methyl butanoic acid were above their sensory perception limits (SPL) of 10 and 0.13 mg/kg, respectively (Belitz et al., 2004). The fatty acids may arise from autoxidation of saturated lipids constituents of fruits, whose production was increased with the thermal treatment (Belitz et al., 2004).

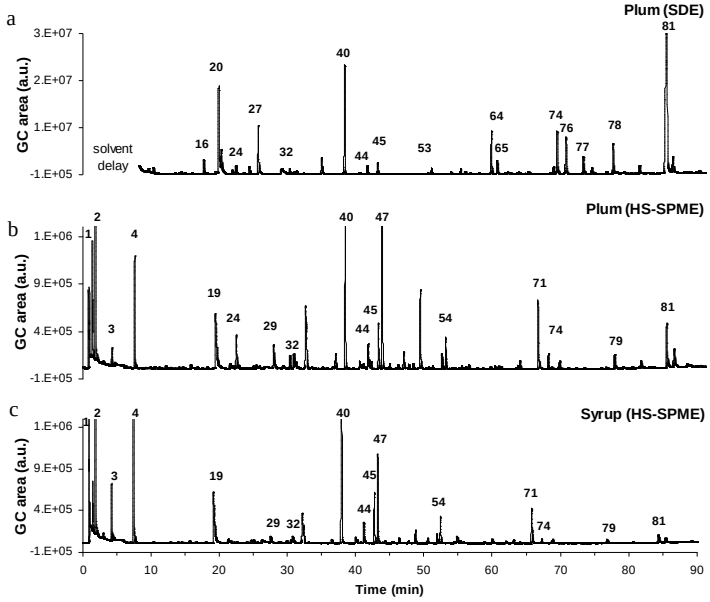

Fig. 1. Total ion chromatograms of the volatile compounds from "Ameixa d'Elvas" plums obtained by a) SDE and b) HS-SPME and from c) sucrose syrup by HS-SPME (attribution of peak numbers of the main components shown in Table 1). a.u. - arbitrary units.

The esters, accounting for 13.5 mg/kg of pulp, represented 27% of the total compounds. 2-Phenylethyl acetate (7.6 mg/kg) was the most abundant of the 16 esters recovered, and this compound was highly above its SPL (0.48 mg/kg) (Pino & Mesa, 2006). Also above their SPL (2.0, 0.06 and 0.0001 mg/kg, respectively) were ethyl hexadecanoate (2.3 mg/kg), ethyl benzoate (0.3 mg/kg) and ethyl octanoate (0.04 mg/kg) (Belitz et al., 2004; Pino, Marbot, Rosado & Vazquez, 2004). The esters are important aroma constituents of fruits, contributing to the fruity aroma notes. However, the processing promotes their hydrolysis and the fruity notes tend to be attenuated (Ismail et al., 1980b).

Representing 18% of the total compounds identified, and accounting for 8.9 mg/kg of the "Ameixa d'Elvas" plums, 7 furans were identified. Furfural was the major furan compound identified (4.7 mg/kg) and it was present in a concentration above its SPL of 3 mg/kg (Pino et al., 2004). Furans are formed from thermal degradation of sugars by caramelization, and they also arise from the decomposition of polysaccharides (Belitz et al., 2004; Rocha, Coimbra & Delgadillo, 2004), thus their production occurred due to the high temperatures reached during processing of "Ameixa d'Elvas". However, part of this furan fraction may also occur as an artefact of the SDE methodology (Rocha et al., 1996). The use of an extraction methodology that does not include a thermal treatment, such as the SPME, can contribute to evaluate what are the compounds that can be considered mainly as SDE artefacts. Furan compounds were detected in some plum products that were submitted to high temperatures, as prunes, canned plums, and jam (Crouzet et al., 1990).

Table 1 - Volatile composition of "Ameixa d'Elvas" plum identified by SDE and HS-SPME, and sucrose syrup by HS-SPME, grouped by chemical classes.

Peak number	Compound	Identif.[a]	Plum		Syrup	SPL[b] (µg/kg)
			SDE (µg/kg of pulp)	HS-SPME (Area x 10^{-6})	HS-SPME (Area x 10^{-6})	
	Acids					
19	acetic acid	A, B, C	80.6 ± 9.4 [c]	73.5 ± 3.9 [d]	80.3 ± 2.3 [d]	60000
34	3-methyl butanoic acid	B, C	179.3 ± 5.9	tr[e]	tr	130
43	hexanoic acid	A, B, C	129.3 ± 15.1	–	–	3000
50	2-ethyl hexanoic acid	B, C	13.2 ±1.9	–	2.2 ± 0.3	–
53	octanoic acid	A, B, C	338.9 ± 39.5	2.9 ± 0.1	7.1 ±1.5	3000)
58	2,4-hexadienoic acid	B, C	–	1.0 ± 0.1	2.5 ± 0.2	–
60	nonanoic acid	A, B, C	188.3 ± 19.6	1.4 ± 0.1	2.3 ± 0.2	3000
66	decanoic acid	A, B, C	822.5 ± 112.8	–	6.6 ± 0.4	10000
71	benzoic acid	B, C	21.8 ± 2.0	61.0 ± 3.2	36.4 ± 2.0	–
74	dodecanoic acid	A, B, C	2404 ± 308	1.5 ± 0.4	2.5 ± 0.3	10000
79	tetradecanoic acid	B, C	1474 ± 153	72.0 ± 2.5	5.3 ± 0.7	10000
80	pentadecanoic acid	B, C	461.1 ± 38.0	–	–	10000
81	hexadecanoic acid	B, C	16484 ± 2648	49.5 ± 3.6	8.8 ± 0.5	10000
	Subtotal (µg/kg or Area x 10^{-6})		***22596.6***	***262.8***	***154.0***	
	Subtotal (%)		***45.7***	***27.6***	***15.5***	
	Alcanes					
41	octadecane	B, C	23.1 ± 2.8	–	–	–
46	nonadecane	B, C	47.2 ± 3.7	–	–	–
	Subtotal (µg/kg or Area x 10^{-6})		***70.3***	**–**	**–**	
	Subtotal (%)		***0.1***	**–**	**–**	
	Alcohols					
2	ethanol	B, C	–	144.4 ± 15.8	167.3 ± 8.0	10
3	2-methyl-1-propanol	B, C	–	9.1 ± 0.5	22.3 ± 4.2	–
4	3-methyl-1-butanol	B, C	22.4 ± 2.2	58.1 ± 3.8	100.2 ± 7.1	300
8	3-methyl-2-buten-1-ol	B, C	16.6 ± 2.3	–	–	3
12	1-hexanol	A, B, C	41.7 ± 4.7	–	0.8 ± 0.1	700
13	3-ethoxy-1-propanol	B, C	31.5 ± 4.5	–	–	–
15	2-butoxyethanol	B, C	38.2 ± 7.3	–	–	–
26	1-octanol	A, B, C	43.5 ± 5.6	2.8 ± 0.1	3.5 ± 0.6	200
28	2,3-butanediol	A, B, C	–	1.0 ± 0.1	3.3 ± 0.2	3
44	benzyl alcohol	A, B, C	458.6 ± 67.7	20.5 ± 2.3	31.2 ± 6.0	20000
45	2-phenylethanol	A, B, C	424.6 ± 71.6	36.5 ± 1.7	66.3 ± 9.4	1100
67	3,7,11,15-tetramethyl-1-hexadecen-3-ol	B, C	44.0 ± 4.8	–	–	–
	Subtotal (µg/kg or Area x 10^{-6})		***1121.3***	***272.4***	***394.9***	
	Subtotal (%)		***2.3***	***28.6***	***39.8***	
	Aldehydes					
1	acetaldehyde	B, C	–	8.4 ± 0.3	3.4 ± 1.0	20
7	2-heptenal	B, C	19.4 ± 2.2	1.6 ± 0.1	–	13
14	nonanal	B, C	85.1 ± 8.9	2.6 ± 0.2	2.3 ± 0.3	1
21	decanal	B, C	–	6.9 ± 0.5	6.4 ± 0.9	0.1
24	benzaldehyde	A, B, C	728.2 ± 57.8	31.3 ± 2.0	tr	350
30	2,6,6-trimethyl-1,3-cyclohexadiene-1-carboxaldehyde	B, C	21.6 ± 2.7	–	–	–
31	phenylacetaldehyde	B, C	1378 ± 133	–	–	4
	Subtotal (µg/kg or Area x 10^{-6})		***2232.3***	***50.8***	***12.1***	
	Subtotal (%)		***4.5***	***5.3***	***1.2***	

Table 1 - Volatile composition of "Ameixa d'Elvas" plum (continuation).

Peak number	Compound	Identif.[a]	Plum		Syrup	SPL[b] (μg/kg)
			SDE (μg/kg of pulp)	HS-SPME (Area x 10^{-6})	HS-SPME (Area x 10^{-6})	
	Esters					
11	ethyl 2-hydroxypropanoate	B, C	40.9 ± 3.4	–	–	–
18	ethyl octanoate	A, B, C	40.1 ± 6.5	1.6 ± 0.1	1.6 ± 0.2	0.1
32	ethyl benzoate	B, C	307.9 ± 47.2	12.3 ± 1.7	3.5 ± 0.3	60
37	benzyl acetate	B, C	16.9 ± 3.2	–	–	360
38	methyl 2-hydroxybenzoate	B, C	22.1 ± 3.4	–	–	–
40	2-phenylethyl acetate	B, C	7635 ± 897	129.9 ± 11.8	277.8 ± 14.7	480
42	ethyl dodecanoate	B, C	81.6 ± 9.4	–	–	5900
52	ethyl tetradecanoate	B, C	67.3 ± 10.8	–	–	4000
62	methyl hexadecanoate	B, C	67.3 ± 8.4	–	–	–
64	ethyl hexadecanoate	B, C	2320 ± 224	–	–	2000
65	dihydromethyl jasmonate	B, C	18.3 ± 2.3	1.1 ± 0.2	0.6 ± 0.2	–
70	diethyl 1,2-benzenedicarboxylate	B, C	109.7 ± 16.1	–	–	–
73	ethyl heptadecanoate	B, C	104.5 ± 12.7	–	–	–
76	ethyl linoleate	B, C	1723 ± 208	–	–	–
77	ethyl linoleolate	B, C	779.6 ±84.2	–	–	–
78	dibutyl 1,2-benzenedicarboxylate	B, C	133.8 ± 13.3	–	–	–
	Subtotal (μg/kg or Area x 10^{-6})		***13467.6***	***144.9***	***283.5***	
	Subtotal (%)		***27.2***	***15.2***	***28.6***	
	Furans					
5	dihydro-2-methyl-3(2H)-furanone	B, C	141.4 ± 6.8	–	–	–
16	5-methyl-2(3H)furanone	B, C	771.4 ± 12.4	–	–	–
20	furfural	A, B, C	4741 ± 496	–	–	3000
22	2,3,4-trimethylfuran	B, C	51.6 ± 5.6	–	–	–
23	1-(2-furanyl)-ethanone	B, C	260.7 ± 44.3	1.4 ± 0.2	0.9 ± 0.1	–
27	5-methylfurfural	A, B, C	2777 ± 347	2.0 ± 0.1	–	–
29	dihydro-2(3H)-furanone	A, B, C	–	28.2 ± 1.4	6.4 ± 0.3	–
33	furfuryl alcohol	A, B, C	122.5 ± 11.8	4.2 ± 0.3	6.4 ± 0.2	–
56	methyl 3-furancarboxylate	B, C	–	2.0 ± 1.0	–	–
75	5-hydroxymethylfurfural	B, C	–	8.0 ± 0.4	4.8 ± 0.2	–
	Subtotal (μg/kg or Area x 10^{-6})		***8865.9***	***45.8***	***18.5***	
	Subtotal (%)		***17.9***	***4.8***	***1.9***	
	Ketones					
6	3-hydroxy-2-butanone	A, B, C	117.0 ± 12.5	–	–	–
9	2,3-octanedione	B, C	19.9 ± 3.6	–	–	–
10	6-methyl-5-hepten-2-one	B, C	17.6 ± 3.9	1.2 ± 0.2	0.8 ± 0.1	50
48	β-ionone	A, B, C	59.3 ± 7.7	-	–	0.007
63	2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	B, C	–	1.9 ± 0.1	–	–
72	benzophenone	B, C	11.1 ± 0.6	–	–	–
	Subtotal (μg/kg or Area x 10^{-6})		***224.8***	***3.1***	***0.8***	
	Subtotal (%)		***0.5***	***0.3***	***0.1***	
	Lactones					
57	γ-decalactone	B, C	39.1 ± 5.3	–	–	11
68	γ-dodecalactone	B, C	71.7 ± 9.5	-	–	7
	Subtotal (μg/kg or Area x 10^{-6})		***110.8***	–	–	
	Subtotal (%)		***0.2***	-	-	
	Phenols					
47	2,6-bis(1,1-dimethylethyl)-4-methylphenol	A, B, C	15.4 ± 1.6	150.8 ± 10.2	98.8 ± 17.2	–
49	2,3,6-trimethylphenol	B, C	21.9 ± 2.9	–	–	–
51	phenol	A, B, C	13.6 ± 1.9	4.5 ± 0.1	–	5900
55	2,6-bis(1,1-dimethylethyl)-4-ethylphenol	B, C	–	3.8 ± 0.2	2.3 ± 0.2	–
59	2-methoxy-4-(2-propenyl)phenol	A, B, C	278.4 ± 35.5	2.2 ± 0.3	5.3 ± 0.5	6
61	4-vinyl-2-methoxy-phenol	B, C	78.5 ± 12.3	–	–	3
69	2,4-bis(1,1-dimethylethyl) phenol	B, C	50.6 ± 5.8	–	4.9 ± 0.9	–
	Subtotal (μg/kg or Area x 10^{-6})		***458.5***	***161.3***	***111.3***	
	Subtotal (%)		***0.9***	***16.9***	***11.2***	
	Terpenoids					
17	*cis*-linalool oxide	A, B, C	71.0 ± 11.3	–	–	320
25	linalool	A, B, C	66.7 ± 5.1	1.7 ± 0.1	2.5 ± 0.3	6
35	α-terpineol	A, B, C	66.7 ± 4.2	–	2.3 ± 0.3	330
39	β-citronellol	A, B, C	10.8 ± 1.0	tr	1.2 ± 0.2	10
	Subtotal (μg/kg or Area x 10^{-6})		***215.1***	***1.7***	***6.0***	
	Subtotal (%)		***0.4***	***0.2***	***0.6***	
	Others					
36	1,2-dihydro-1,1,6-trimethylnaphthalene	B, C	38.8 ± 8.1	–	–	–
54	2-formyl-1-methylpyrrole	B, C	27.9 ± 3.4	12.1 ± 1.4	11.0 ± 2.4	–
	Subtotal (μg/kg or Area x 10^{-6})		***66.7***	***12.1***	***11.0***	
	Subtotal (%)		***0.1***	***1.3***	***1.1***	
	TOTAL		**49430**	**955**	**992**	

[a] The reliability of the identification or structural proposal is indicated by the following: A, mass spectra and retention time consistent with that of a standard; B, structural proposal given on the basis of mass spectral data (Wiley 275); C, mass spectrum consistent with spectra found in the literature. [b] Sensory perception limit reported in the literature. [c] Mean of six replicates (± standard deviation). [d] Mean of five replicates (± standard deviation). [e] tr- trace, detected by ion extraction analysis mode using specific *m/z*.

Aldehydes accounted for 2.2 mg/kg of pulp, representing 4.5% of the total compounds quantified. Phenylacetaldehyde and benzaldehyde (1.4 and 0.7 mg/kg, respectively) were the most abundant aldehydes recovered. These compounds were above their SPL (0.004 and 0.35 mg/kg, respectively) (Belitz et al., 2004; Pino et al., 2004); also

above their SPL were 2-heptenal (0.02 mg/kg) and nonanal (0.09 mg/kg), 0.01 and 0.001 mg/kg, respectively (Pino et al., 2006). Benzaldehyde has been already reported to increase on heating of fruits and/or making of jams (Belitz et al., 2004).

Alcohols, phenols, ketones, and alkanes accounted, respectively, for 1.1, 0.5, 0.2, and 0.1 mg/kg of pulp, representing together 3.8% of the total compounds. However, within these groups of compounds, 2-methoxy-4-(2-propenyl)phenol (0.3 mg/kg), 4-vinyl-2-methoxy-phenol (0.08 mg/kg), β-ionone (0.06 mg/kg), and 3-methyl-2-buten-1-ol (0.02 mg/kg) were above their SPL (0.006, 0.003, 0.000007, and 0.003 mg/kg, respectively) (Belitz et al., 2004; Pino et al., 2004; Pino et al., 2006). The origin of C_6 alcohols, such as 1-hexanol, reported as an important contributor to the aroma of the fresh plums (Crouzet et al., 1990; Williams et al., 1981), are related to the lipoxygenase activity. This enzyme that occurs in plants and, namely, in fruits catalyzes the oxidation of unsaturated fatty acids (Belitz et al., 2004), as a first step to the production of compounds such as the short chain alcohols. The C_6 alcohols contribute to green and herbaceous notes. Some phenols have been also related with the heat processed aroma of the plums. Their origin was explained by thermal degradation of precursors like phenolic acids (Crouzet et al., 1990; Ismail et al., 1980b; Sabarez, Price & Korth, 2000).

Terpenoids accounted for 0.2 mg/kg, representing only 0.4% of the total compounds identified. From these, linalool (0.07 mg/kg) was above its SPL (0.006 mg/kg). In this group, β-citronellol was recovered in a concentration similar to its SPL of 0.01 mg/kg (Belitz et al., 2004). The terpenoids, namely the monoterpenols, were reported as volatiles components of fruits responsible for a wide spectrum of aromas, mostly perceived as very pleasant (Belitz et al., 2004; Coelho, Rocha, Delgadillo & Coimbra, 2006). These compounds are responsible to the varietal character of the fruits, being present, at least in part, as glycosides (Belitz et al., 2004). Linalool, already identified in fresh plums (Crouzet et al., 1990; Williams et al., 1981), as well as *cis*-linalool oxide, α-terpineol, and β-citronellol may arise, at least in part, due to the hydrolysis of the corresponding glycosides promoted by the heat processing.

γ-Dodecalactone and γ-decalactone, which accounted for 0.07 mg/kg and 0.04 mg/kg, were above its SPL of 0.007 mg/kg and 0.01 mg/kg, respectively (Engel, Flath, Buttery, Mon, Ramming & Teranishi, 1988). Lactones are formed from the corresponding hydroxy

acids (Belitz et al., 2004). These compounds, particularly γ-lactones, are important compounds in terms of their contribution to the aroma and, in general, present fruity odour descriptors.

3.2. Establishment of the headspace volatile profile of "Ameixa d'Elvas"

3.2.1. Headspace volatile composition of "Ameixa d'Elvas" plums

In order to determine the headspace volatile profile of "Ameixa d'Elvas", SPME technique was used. Figure 1 shows the total ion chromatogram of volatile compounds for "Ameixa d'Elvas" plums obtained by SPME (Fig. 1b). Forty compounds were identified as shown in Table 1. Excluding the alkanes, compounds representative of all the other chemical groups detected by SDE were also detected by HS-SPME. Alcohols (29% of the compounds) and acids (28%) represented the principal chemical groups.

A qualitative comparison between the SDE and HS-SPME data indicates that the higher differences were observed for the esters, furans, ketones, and terpenoids, present in higher number in the SDE data set. These results may be explained based on the peculiarities of each extraction procedure used. The SPME is a non-exhaustive extraction methodology that also combines the sampling and pre-concentration steps. The SPME experimental parameters should be optimized and controlled to guarantee that a proportional relationship is obtained between the amount of the analyte extracted by the SPME fibre and its initial concentration in the sample matrix (Kleeberg, Dobberstein, Hinrichsen, Müller, Weber & Steinhart, 2007). The SDE represents an exhaustive extraction methodology. In this case, the plum was dispersed as a pulp in hot water and extracted with dichloromethane for 3 h promoting a high area of contact with the solvent. Consequently, an extraction of a higher number of compounds was obtained than using SPME. Furthermore, the conditions of the SDE analysis, namely the temperature, may provoke artefacts. For example, β-ionone, a ketone, is a C_{13}norisoprenoid compound that arises from carotenoids. It has been reported for other fruits, such as grapes, that during ripening of the fruit glycosylation of norisoprenoids may occur. Therefore, a decrease of these compounds in free form was expected (Baumes, Wirth, Bureau, Gunata &

Razungles, 2002). However, the results obtained suggest that during the SDE, the reactions originating the β-ionone (hydrolysis of the glycosidic precursor, reduction of *Grasshopper ketone*, dehydration…) (Belitz et al., 2004) have occurred. This may explain the recovery of β-ionone by SDE and its absence in the headspace characterized by HS-SPME. In what concerns the furans, it was assumed that the compounds recovered only by the SDE, such as dihydro-2-methyl-3(2H)-furanone, 5-methyl-2(3H)furanone, furfural, and 2,3,4-trimethylfuran were formed mainly during the SDE extraction, and were considered as SDE artefacts. The other furans recovered by SDE and also present in the headspace, given by the HS-SPME data, may have been produced due to the high temperatures reached during the candying process of "Ameixa d'Elvas".

On the other hand, the HS-SPME allows the detection of the low boiling point volatiles that overlap with the solvent in the SDE. Thus, acetaldehyde, ethanol, and 2-methyl-1-propanol could only be detected by HS-SPME, since they appear in the retention times that are included in the solvent delay. The occurrence of ethanol led to the possibility that fermentation had occurred. This hypothesis is also suggested by the presence of 2-methoxy-4-(2-propenyl)phenol (eugenol), acetic acid, and several ethyl esters quantified by SDE. Eugenol arised from the corresponding phenolic alcohol or from ferulic acid during fermentation (Crouzet et al., 1990), compounds also found in fermented plum brandies (Ismail, Williams & Tucknott, 1980a).

In the HS-SPME analysis, the "Ameixa d'Elvas" plum was cut into two halves and the intact plum was analyzed. This allowed to simulate the state of the plum when it is consumed, releasing only the volatiles that potentially contribute to its aroma as are perceived by the consumers. As the intact plum may retain the volatiles, not being released to the headspace, the number of compounds (even the quantity, but this was not the aim of the present study) that may be recovered in the headspace should be lower than that obtained by SDE.

3.2.2. Headspace volatile composition of "Ameixa d'Elvas" sucrose syrup

In order to evaluate the contribution of the 75 ºBrix syrup used in the storage of "Ameixa d'Elvas" plums to the overall aroma of the product, it was analyzed by HS-SPME using the experimental conditions already used for the analysis of the plums headspace.

Considering the chemical composition of the syrup, the SDE should not be adequate to its analysis, since it promotes a high level of furans.

Figure 1c shows the total ion chromatogram of the volatile compounds for "Ameixa d'Elvas" syrup. In "Ameixa d'Elvas" syrup 40 compounds were identified (Table 1). All the chemical groups detected in the plum by HS-SPME were also detected in the syrup. Alcohols and esters represented the principal chemical groups, with 40% and 29% of the compounds, respectively. Although with different relative GC peak areas, generally, the compounds identified in the fruits were also identified in the syrup (Table 1), indicating the diffusion of the volatile components from the fruits to the syrup during their processing and storage. It is also important to pointed out that in the syrup it was detected more terpenoids (linalool, α-terpineol, and β-citronelol) than in the fruit headspace, which suggests the capacity of the syrup to retain and concentrate these compounds. As the fruit can also be consumed with the syrup, this result advises its potential contribution to the preservation of the aroma character of "Ameixa d'Elvas".

3.3. Estimation of the contribution of the "Ameixa d´Elvas" volatiles to the aroma

3.3.1. Identification of the compounds with aroma indexes higher than 1

Calculations of aroma indexes (*I*) are necessary to obtain a more accurate estimation of the contribution of the compounds to the aroma of "Ameixa d'Elvas". For this purpose, the aroma potential of each compound was assessed by calculating the aroma index ($I=c/s$ where c is the concentration found in the plum pulp by SDE and s is the SPL for the compound reported in literature).

The SPL vary depending on the sample matrix, pH, sample temperature, and the methodologies of sensory analysis used. Therefore, comparisons of aroma contribution on *I* are very difficult when SPL from different sources are used. However in this study SPL in water were used for all the compounds available because there is no data available for candied fruit matrices, although, ideally, the SPL should have been determined for "Ameixa d'Elvas" (Grosch, 2001; Qian & Reineccius, 2003). The use of SPL determined in a model medium (water) ignores the influence of the fruit components on the release of

odourants. Despite these limitations, the *I* concept is still considered a very useful tool in aroma research studies (Belitz et al., 2004; Stephan, Bücking & Steinhart, 2000).

Compounds that exhibit *I>1* are considered to have a potential individual contribution to the aroma. Furthermore, according to Meilgaard´s suggestion of the sensory contribution to the overall aroma of a substance, when its concentration is at least 20 % of the threshold unit (*I*>0.2), it should be considered (Belitz et al., 2004).

In "Ameixa d'Elvas", 19 compounds were identified as having an *I>1* (Table 2):

- β-ionone was found as having the highest aroma index value (*I*=8429), and exhibit a violet-like odour, although it is also associated with sweet, fruity and berry descriptors in tropical fruits (Mahattanatawee, Goodner & Baldwin, 2005; Pino et al., 2006);

- ethyl octanoate (*I*=400), 2-phenylethyl acetate (*I*=16), ethyl benzoate (*I*=5) and ethyl hexadecanoate (*I*=1) have been described to have aroma descriptors of, respectively, pleasant cooked fruity (Fang & Qian, 2005), fruity rosy, fruity (Pino et al., 2006), and peppery (Chisholm, Wilson & Gaskey, 2003);

- 3-methyl-2-buten-1-ol was the only alcohol detected that exhibits an aroma index >1 (*I*=6) and it may contribute individually with apple notes (Boulanger et al., 2000);

- γ-dodecalactone had an *I* of 10 and it is associated with peach and apricot descriptors (Engel et al., 1988), and γ-decalactone, with an *I* of 4, is associated with peach, apricot, and nectarine descriptors (Engel et al., 1988);

- furfural was detected with an *I* of 2, which could contribute individually with toast and burnt notes (Fang et al., 2005);

- nonanal (*I*=85) and benzaldehyde (*I*=2) have been described as contributors to the aroma of the fresh plums (Williams et al., 1981), as well as major constituents of canned plums and jams. All these compounds, as well as 2-heptenal (*I*=2), are associated with sweet, fruity, and floral odours (Mahattanatawee et al., 2005). In addition, phenylacetaldehyde, which showed a high aroma index (*I*=345) has been described as having a green pungent odour (Mahattanatawee et al., 2005);

- linalool (*I*=11) and β-citronellol (*I*=1) exhibit sweet, floral and fruity odours (Fang et al., 2005);

- 2-methoxy-4-(2-propenyl)phenol (*I*=46) and 4-vinyl-2-methoxyphenol (*I*=26), have aroma descriptors of, respectively, sweet phenolic (Miranda, Nogueira, Pontes & Rezende, 2001; Sabarez et al., 2000) and clove-like (Belitz et al., 2004);

- hexadecanoic acid (*I*=2) has been described to have grassy and heavy descriptors (Boulanger et al., 2000), and 3-methyl butanoic acid (*I*=1) and been described as harsh and pungent in fruits (Miranda et al., 2001; Sabarez et al., 2000).

Table 2 - Aroma index (*I*) of the volatile compounds of "Ameixa d'Elvas" plum with *I*>1 and their odour descriptor (from literature).

Compound	*I*	*Odour descriptor*	*HS-SPME*	
			Plum	*Syrup*
β-ionone	*8429*	violet, sweet, fruity, berry	—	—
ethyl octanoate	*400*	pleasant, cooked, fruity	x	x
phenylacetaldehyde	*345*	green, pungent	—	—
nonanal	*85*	sweet, fruity	x	x
2-methoxy-4-(2-propenyl)phenol (eugenol)	*46*	sweet, phenolic	x	x
4-vinyl-2-methoxy-phenol	*26*	clove-like	—	—
2-phenylethyl acetate	*16*	fruity, rose	x	x
linalool	*11*	sweet, floral	x	x
γ-dodecalactone	*10*	peach, apricot	—	—
3-methyl-2-buten-1-ol	*5.5*	apple	—	—
ethyl benzoate	*5.1*	fruity	x	x
γ-decalactone	*3.6*	peach, apricot, nectarine	—	—
benzaldehyde	*2.1*	sweet, fruity	x	x
2-heptenal	*1.9*	sweet, fruity	x	—
hexadecanoic acid	*1.6*	grassy, heavy	x	x
furfural	*1.6*	toasty, burnt	—	—
3-methyl butanoic acid	*1.4*	harsh, pungent	x	x
ethyl hexadecanoate	*1.2*	peppery	—	—
β-citronellol	*1.1*	fruity	x	x

3.3.2. Searching the would-be impact odourants

HS-SPME headspace technique was used to evaluate if the volatile compounds identified as having *I>1* were present in the headspace of the plums. As the molecules present in the headspace are indeed responsible for the smell that is perceived by the olfactory system, if they are in concentrations above their SPL, these compounds are considered to be potential contributors to the aroma of the "Ameixa d'Elvas" and can be designed as would-be impact odourants.

From the 19 compounds that exhibit an *I*>1, 11 were detected in "Ameixa d'Elvas" plums by HS-SPME: ethyl octanoate, nonanal, 2-methoxy-4-(2-propenyl)phenol, 2-phenylethyl acetate, linalool, ethyl benzoate, benzaldehyde, 2-heptenal, hexadecanoic acid, 3-methyl butanoic acid, and β-citronellol (Table 2). Therefore, these are the would-be impact odourants of "Ameixa d'Elvas". Almost all these compounds are associated with sweet, cooked and fruity odours (Table 2). The other 8 compounds determined to have an

I>1 and that were not detected in the fruit headspace by HS-SPME were probably retained in the fruit, not being released to the headspace of the "Ameixa d'Elvas".

Excluding the 2-heptenal, all the established would-be impact odourants of "Ameixa d'Elvas" were also detected in the syrup headspace (Table 2). Retention of volatile compounds, namely linalool, has been shown to occur in sucrose/pectin-rich matrices, although no interaction has been observed for ethyl esters (Savary, Guichard, Doublier & Cayot, 2006). Also, high methoxyl pectins have been shown to cause a decrease in the amount of volatile compounds detected in the headspace in jams (Guichard, Issanchou, Descourvieres & Etievant, 1991). "Ameixa d'Elvas" syrup is composed by 1% polysaccharides, mostly pectic polysaccharides with a mean degree of esterification of 68% (results not shown). This allows expecting retention of volatiles in the sucrose syrup matrix. However, syrup could have an opposite effect, because increasing concentrations of sucrose enhance the release of volatile compounds to the headspace (Nahon, Harrison & Roozen, 2000; Nahon, Koren, Roozen & Posthumus, 1998). The release of the compounds to the syrup headspace depends on the equilibrium between these effects, as well as on the physico-chemical properties of the compounds.

4. Concluding remarks

The present study allowed to establish the volatile composition of "Ameixa d´Elvas", which includes 10 chemical groups: acids, esters, furans, aldehydes, alcohols, phenols, ketones, terpenoids, lactones, and alkanes. This composition reflects the complexity of the reactions (enzymatic or not) and rearrangements that happen during the fruit ripening plus those occurring due to the thermal processing of the fruit in the presence of sucrose. "Ameixa d´Elvas" contains volatile compounds that arise from different origins: i) characteristic from the fruit (acids, terpenoids, lactones, and esters), ii) produced during the heat processing (furans and compounds released from the glycosidic precursors), and iii) compounds that seems to indicate the occurrence of fermentation (ethanol, eugenol, esters, and acetic acid). Some compounds have more than one origin.

The approach applied to obtain these results included the use of: i) SDE, that promoted an exhaustive extraction of the pulp dispersed in hot water, allowing a deep

analysis of the volatile fraction of "Ameixa d´Elvas" and the determination of the compounds with *I*>1, and ii) HS-SPME, allowing to detect the volatile compounds present in the headspace of the intact pulp, and potentially responsible for the aroma properties. From the 19 compounds that exhibit an *I*>1, 11 compounds were detected in "Ameixa d'Elvas" plums by HS-SPME: ethyl octanoate, nonanal, 2-methoxy-4-(2-propenyl)phenol, 2-phenylethylacetate, linalool, ethyl benzoate, benzaldehyde, 2-heptenal, hexadecanoic acid, 3-methyl butanoic acid, and β-citronellol. Therefore, these are the would-be impact odourants of "Ameixa d'Elvas", which are associated with sweet, cooked, and fruity odours. Excluding 2-heptenal, all the established would-be impact odourants of "Ameixa d´Elvas" were also detected in the syrup headspace. The present study also indicated the occurrence of transference of volatile components from the processed fruits to the syrup during storage, revealing the capacity of the syrup to retain and concentrate these compounds. As the fruit can also be consumed with the syrup, this result advises to its potential contribution to the preservation of the aroma character of "Ameixa d'Elvas" candied plums.

This methodology that comprises the combination of the analysis of SDE-GC-qMS and HS-SPME-GC-qMS data seems to be suitable to characterize the volatile fraction as well as to estimate its contribution to the aroma properties of heat treated fruits. However, as a preliminary study, these features need confirmation using a sensory panel and/or GC-olfactometry. The approach proposed in this study can be extended to the analysis of other solid matrices.

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EFFECT OF CANDYING ON MICROSTRUCTURE AND TEXTURE OF PLUMS (*PRUNUS DOMESTICA* L.)

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Effect of candying on microstructure and texture of plums (*Prunus domestica* L.)*

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Abstract

The plums (*Prunus domestica* L.) of a specific 'Green Gage' variety, ''Rainha Cláudia Verde'', are used to produce the candied product ''Ameixa d'Elvas''. The candying process consists in boiling the intact plums in water and further immersion in sucrose syrup until 75 ºBrix. Scanning electron microscopy of unprocessed plums revealed perfectly turgid parenchyma isodiametric cells with few intercellular spaces and the boiled plums showed a loss of intercellular adhesion of parenchyma cells and ruptures on the vascular strand structure. However, candied plums showed recovery of the turgidity and cell-to-cell adhesion of parenchyma cells. Texture analysis showed that the unprocessed plums had a fairly hard texture, improved by the presence of the skin. Firmness, rigidity, and deformation work had a sharp decrease upon boiling. However, after candying, an increase of firmness and deformation work was observed, also improved by the skin. Microstructure and texture recovery of the candying product suggests the formation of a jam-like structure in the middle lamella region promoted by the syrup. Nevertheless, cell adhesion recovery is limited by the extent of the disruption of the tissues during the boiling process. This is the first report concerning texture and microstructure changes of plums occurring during the candying process.

Keywords: Plum, Ameixa d'Elvas; 'Green Gage'; Osmotic dehydration; Scanning electron microscopy; Fruit firmness; Fruit rigidity

1. Introduction

The primary objective of food processing is the preservation of perishable foods in a stable form that can be stored for consumption after the production period. Heat processing is the main pre-treatment required for food preservation by the food industry and its goal is to render foods microbiologically safe for consumption and to prevent the action of deleterious enzymes on food quality. The flesh is the edible part of the fruits and is mainly composed of thin-walled parenchymatous cells composed by pectic polysaccharides, cellulose, other polysaccharides generally called hemicelluloses, and minor components such as structural proteins and phenolic compounds (Carpita & Gibeaut, 1993; Vincken et al., 2003). Heat processing modifies the texture of fruits, partly due to the turgor loss caused by cell membrane disruption, but also due to a variety of chemical and biochemical changes in the cell wall polysaccharides (Mafra, Barros, & Coimbra, 2006; Mafra, Barros, & Coimbra, 2007; Waldron, Smith, Parr, Ng, & Parker, 1997). Fruit tissue softening occurs as a result of an increase in cell separation and/or cell wall weakening. Middle lamella is heat labile and its dissolution results in the separation of cell walls (Van Buren, 1979).

Candying of fruits causes a reduction of water activity, by osmotic dehydration, which slows down deteriorative reactions

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and increases fruits microbial stability, thus prolonging fruits shelf-life. The complex microstructure of the fruits influences the candying process, since water and solutes leave the interior of the cells and sucrose diffuses to and penetrates the free spaces of the cell structure (Peiro´, Dias, Camacho, & Martinez-Navarrete, 2006; Quiles, Perez-Munuera, Hernando, & Lluch, 2003). Changes on cell walls microstructure are the main contributing factor to the modification of the textural properties, including cellular turgor and cell wall integrity. Many aspects of fruits cell structure and mechanical properties are affected during osmotic dehydration with sucrose solutions, such as alteration of cell walls, splitting of the middle lamella, lysis of membranes (plasmalemma and tonoplast), and tissue shrinkage (Nieto, Salvatori, Castro, & Alzamora, 1998). Improvements on the quality of osmotically dried food products could be based on interventions at the microscopic level, such as on plant cells, namely at the cell walls level and their polymers network (Aguilera, 2005; Quiles, Hernando, Perez-Munuera, & Lluch, 2007). For this, it is necessary to have a better knowledge and understanding of the changes that occur at the microscopic and molecular level upon candying.

''Ameixa d'Elvas'' is a ''Protected Designation of Origin'' recognized by the European Union for a candied plum from a delimited region of Alto Alentejo (South East of Portugal) obtained by a traditional and characteristic candying process. Only the fruits of a special type of 'Green Gage' plum (*Prunus domestica* L.) variety, ''Rainha Cláudia Verde'', can be utilized to produce ''Ameixa d'Elvas''. The candying process consists of boiling the intact plums in water for 15 min and further immersion in sugar syrup, which is successively concentrated until 75 ºBrix, for osmotic dehydration. The peculiar texture of these candied plums is an important sensory property for consumer acceptance. However, no scientific information is yet available concerning texture and microstructure changes of plums occurring during the candying process.

This work describes the microstructural and textural changes of ''Rainha Cláudia Verde'' plums variety during candying from two orchards, Vila Viçosa (VV) and Cano (CA), in the same geographic region and at the same stage of ripening. These two different orchards were selected based on the local knowledge about the different behaviour of the plums during the candying process. Contrarily to VV plums, the fruits from CA show frequent skin disruption and loss of pulp consistency during the boiling step, originating a candied soft product, with poor texture properties, with no commercial value. The present work is a contribution to increase the scientific knowledge about modifications at the microstructure and texture level occurring on plums tissues after the boiling treatment and after the osmotic dehydration with sucrose syrup. This knowledge is needed to adequately control the candying process and improve the quality of candied plums, namely with desirable texture characteristics, which is decisive for plums arising from the CA orchard and other orchards from which the plums show the same texture degradation problems after processing.

2. Experimental

2.1. Plant material and sample preparation

Plums (*P. domestica* L.) of 'Green Gage' variety, ''Rainha Cláudia Verde'', were collected in the precisely established stage of ripening adequate for candying (16-17 ºBrix, pH 3.3, and titratable acidity of 1 meq of malic acid per 100 g of fresh weight) in two orchards, Vila Viçosa (VV) and Cano (CA). VV fruits had an average weight of 42 ± 1 g, while CA fruits had 36 ± 1 g. The candying process was carried out in an industrial plant by boiling the plums in water for 15 min in a tank of 0.7 m^3 capacity using a water:fruit ratio of 4:1. This was followed by immersion of the trays containing the intact boiled plums in storage tanks of 1.5 m^3 filled with 60 ºBrix sucrose syrup (syrup:fruit ratio of 2:1) during one day. The sucrose solution was then concentrated until 65 ºBrix and, after 7 days, until 75 ºBrix. The plums were stored 2 months in the 75 ºBrix sucrose syrup being its concentration occasionally (2-3 times) corrected due to its hygroscopicity.

The microstructure and texture analysis were performed on fresh, boiled, and candied plums. The plums for the analysis were collected in the factory Confibor, Lda. (Estremoz, Portugal), where they were processed, brought immediately to the laboratory, and analysed for microscopy and texture in the same day.

2.2. Scanning electronic microscopy (SEM)

Preparation and fixation of the fresh, boiled, and candied plums were performed as previously described by Pinto, Valentim, Costa, Castro, and Santos (2002). Briefly, the parenchyma tissue of three fruits was cut into pieces (approximately 0.2 x 0.5 x 0.5 cm), on the transversal line in different regions of plums mesocarp. Leaf samples were fixed with 2.0/100 mL glutaraldehyde in 0.04 mol/L of PIPES buffer pH 7.6 (Duchefa, Haarlem, The Netherlands), at 4 ºC for 16 h. Dehydration was achieved by successive immersions in aqueous ethanol solutions of increasing concentration (30–100/100 mL), acetone solutions of increasing concentration (30 – 100/100 mL), and, finally, in a critical point device (Baltec CPD 030, Canonsburg, PA, USA) using CO_2 as transition agent. Samples were fixed on steel supports and coated with gold using a JEOL metalizer (FFC-1100, Tokyo, Japan) at 1100–1200 V, 5 mA for 10 min. Samples were observed in a scanning electron microscope (Hitachi, S4100, Tokyo, Japan) at 20 kV.

2.3. Fruit texture analysis

Puncture tests were performed using a TA-Hdi Texture Analyser (Stable Micro Systems, Godalming, UK), equipped with a 5 kg load cell and a 2-mm diameter cylindrical stainless steel probe. Penetration was done at 1 mm/s to a depth of 15 mm. Each plum was cut on the longitudinal line and one half was analysed with skin and another half without skin, with puncturing carried out from the external plums surface. At least

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six different fruits of each sample were used, and each half was punctured at five different locations, one on the middle and four on the sides of the fruit, 7–10 mm apart from the centre, in the corners of an imaginary square. Firmness (the peak maximum force), rigidity (the maximum initial slope of the curve obtained during the puncture tests), and the work done on the sample during the downstroke puncture, were used to characterise the textural properties of the samples.

2.4. Statistical analysis

Results are presented as mean value and the reproducibility of the results expressed as error bars (standard deviation) in figures. Statistical analysis of the experimental results was based on Student's *t* test (Microsoft Excel, Microsoft Corporation, Redmond, USA) and significant difference was considered at the level of $p < 0.05$.

3. Results and discussion

3.1. Microstructure analysis of fresh, boiled, and candied plums by SEM

Images of transversal section cuts of fresh, boiled, and candied plums obtained by SEM are presented in Fig. 1. Tissues of fresh plums showed isodiametric parenchyma cells with a regular shape. Parenchyma cells of the fruit flesh increased progressively in size with distance from the epidermis (Fig. 1a and b). Tissue fracture caused by cutting involves cell wall breaking. The cells were perfectly turgid with very few intercellular spaces and with an apparently consistent cell wall structure. A well defined middle lamella between parenchyma cells was observed and these small intercellular spaces result from joining three or four cells (Fig. 1c and d). Vascular strands were also observed distributed in the parenchyma tissue, surrounded by smaller parenchyma cells (Fig. 1a and b).

Parenchyma cells of boiled plums showed a clear turgor loss, presenting degradation and some shrinkage in contours of the cell wall (Fig. 1e and f). Many cells presented separation, which should prevent the cell fracture observed in fresh plums, leading to tissues with quite different aspect from those in fresh plum presenting intact separated cells. As a consequence, parenchyma cells of boiled plums showed a more irregular shape and bigger intercellular spaces than those observed in the fresh plum tissues. This turgor loss of parenchyma cells and loss of cell wall strength and adhesion were reported for other heat treated fruits (Ferreira et al., in press; Van Buren, 1979; Waldron et al., 1997). Loss of cell wall adhesion has been explained by alterations in pectic polysaccharides of middle lamella, namely by their solubilisation and degradation. Boiled parenchyma cells from CA plums showed cell walls with more intact cells and an apparent higher cell separation, when compared to VV plums (Fig. 1e and f). The boiling step causes a decrease of the density of the fruit and its floating in water, which is a processing parameter used to establish the end of the thermal treatment.

SEM images of candied plums tissue showed an important reduction in intercellular spaces comparing to boiled plums and cells seem to partially recover their fresh shape (Fig. 1g and h). Tissue fracture caused by cutting involves cell wall breaking, as observed for fresh plums. Cells looked turgid and with round shape similar to cells from fresh plums tissue and the original arrangements of cell walls were rather well maintained. The candying process of plums increased the cell wall contact of parenchyma cells, possibly due to the promotion by the diffused sucrose, of the middle lamella pectic polysaccharides gelling ability, as it occurs in jams (Suutarinen, Honkapaa, Heinio, Autio, & Mokkila, 2000). Microstructure cell recovery of apple tissues after immersion in sucrose solutions has already been reported when long periods of time (2 days) are used, promoting a compositional equilibrium between the concentrated sucrose solution and the fruit (Nieto, Salvatori, Castro, & Alzamora, 2004; Nieto et al., 1998). However, this structure recovery was not observed in apples that were previously heat treated, which was explained by the disruption of membrane permeability integrity (Salvatori & Alzamora, 2000). In the case of candied plums, it seems that the level of middle lamella degradation, caused by the boiling step, is a key factor for the recovery of the original shape of parenchyma cells. By comparison of the plums from the two orchards, it can be shown that VV plums presented smaller intercellular spaces, a more uniform shape, and almost complete recovery of cell wall adhesion compared to CA candied plums. This can be due to the higher middle lamella degradation observed after boiling of CA plums, limiting recovery of cells shape and adhesion.

Vascular strands are formed by xylem and phloem fibres, where phloem surrounds the interior core of xylem (Fig. 2a). Since no differences were observed in the vascular strands between plums from VV and CA orchards, only images of a vascular strand of VV plum tissues were presented in Fig. 2. The secondary lignified cell walls are visible in Fig. 2a, although primary cell walls of xylem cells can be also visible using higher zoom-in amplifications. Although conductive functions are the primary role of vascular strands (water-conducting xylem and nutrient-conducting phloem), these tissues have been shown to also play a very important role in the integrity maintenance and texture characteristics of tissues during processing (Suutarinen et al., 2000; Waldron et al., 1997).

With the thermal processing, fractures in the vascular tissues were observed, namely, separation of the phloem from xylem structures (Fig. 2b). This may occur due to the tension created between these structures owing to the swelling of the parenchyma cells. However, phloem tissue was found, in general, to be less disrupted by the thermal treatment than xylem tissue, an observation similar to that previously reported for blanched carrots (Kidmose & Martens, 1999). After candying, the xylem and phloem structures still showed similar fractures as those observed in boiled tissues (Fig. 2c), do not allowing to observe the recovery of cell adhesion upon candying seen in the parenchyma cells.

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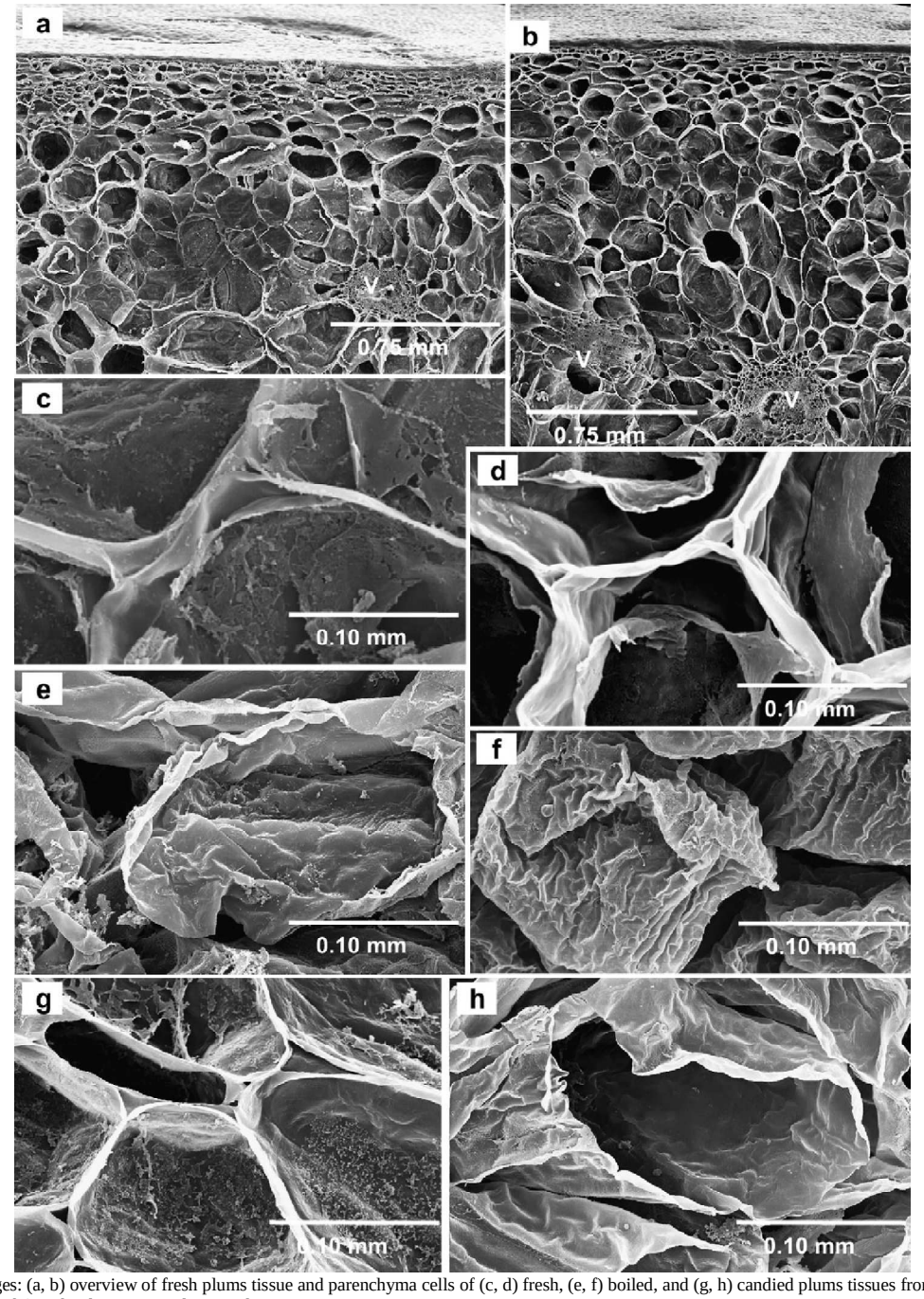

Fig. 1. SEM images: (a, b) overview of fresh plums tissue and parenchyma cells of (c, d) fresh, (e, f) boiled, and (g, h) candied plums tissues from Vila Viçosa (left) and Cano (right) orchards. V – vascular strands.

3.2. Texture analysis of fresh, boiled, and candied plums

Puncture tests are a convenient and commonly used instrumental texture measurement, since they consistently provide a good prediction of a number of texture attributes when compared to other instrumental measurements (Harker et al., 2002; Harker, Stec, Hallett, & Bennett, 1997; Varela, Salvador, & Fiszman, 2007). Examples of typical force-distance curves obtained for puncture tests of fresh, boiled, and candied plums with and without skin are presented in Fig. 3. For all samples analysed, there was a steady increase up to a maximum force as the puncture probe was driven into the fruit, and there was

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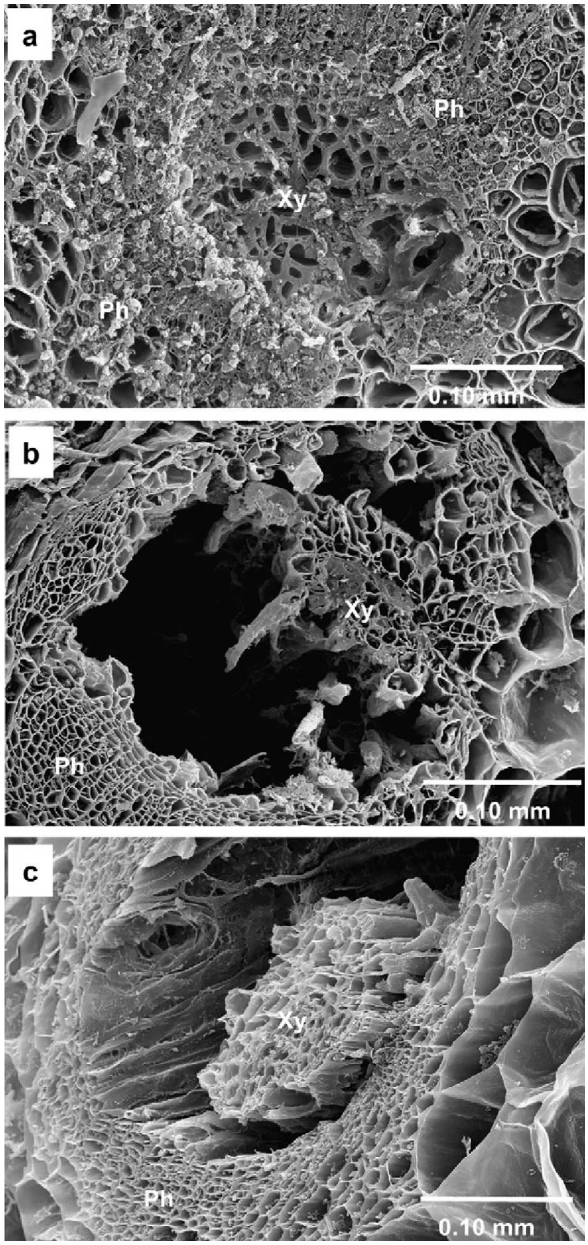

Fig. 2. SEM images of a vascular strand with xylem and phloem cells of (a) fresh, (b) boiled, and (c) candied plums tissues from Vila Viçosa orchard. Xy – xylem; Ph – phloem.

a reduction in the force required to drive the probe further into the fruit flesh following tissue failure. These are the typical force–distance curves associated with moderately hard fruits, the shape of the curve indicating a steady increase in force up to a point when the tissue suddenly fractures (Harker et al., 1997; Varela et al., 2007). This increase in force is higher for the fresh plums analysed with the skin, resulting in a higher maximum force necessary to puncture the plums. After the tissue failure, the force necessary to drive the probe into the flesh

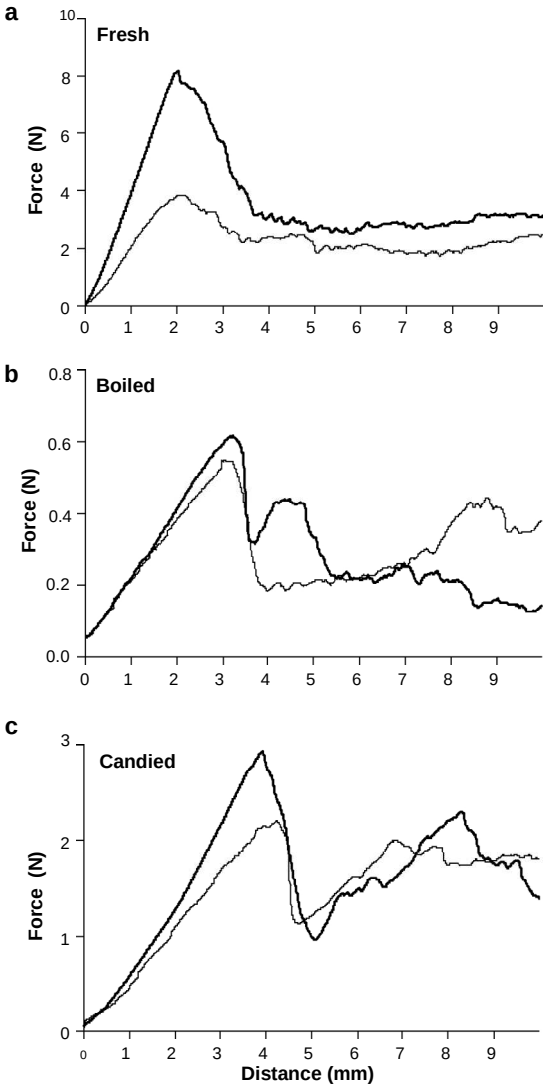

Fig. 3. Force–distance curves obtained for fresh, boiled, and candied plums (Vila Viçosa orchard) with (▬) and without skin (—), using puncture tests.

was similar for the plums with or without skin. Tissue failure in puncture tests, where similar forceedistance curves were obtained, has been associated with breaking of the relatively large cells and the shape of the curves has been attributed to the brittle nature of the relatively thin cell walls (Harker et al., 1997). A similar situation seems to be the case of the plums analysed in this work, since the SEM results indicated the occurrence of parenchymatous cells with thin cell walls in the fresh plums tissue of both orchards (Fig. 1c and d). For boiled and candied plums, the shape of force–distance curves was similar, but the difference between the maximum force needed for the rupture of skin and the force required to drive the probe into the fruit flesh was smaller (Fig. 3b and c). This fact was probably due to modifications of the skin tissue of plums with boiling process, resulting in softening.

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From the force-distance curves, the maximum force, indicative of the firmness of the tissue; the initial slope of the curve, related to the rigidity or the elastic behaviour of the tissue; and the total deformation work done on the tissue until fracture, related to the energy need for the rupture of the tissue, were calculated. The results obtained for VV and CA plums for firmness, rigidity, and deformation work, with and without skin, during the candying process are presented in Fig. 4. The values obtained represent the average of all puncture tests, since no significant differences were found in the puncture tests performed on the middle of the fruits or on the side (around the middle punctured point).

In unprocessed plums, the presence of skin in puncture tests obviously increased the values of firmness, rigidity, and deformation work, since the epidermis tissue of the plums showed to have small cells that were tightly packed, as was observed in SEM images (Fig. 1a and b), a tissue architecture and structure that confers a higher resistance to deformation and fracture (Harker et al., 1997). The results of the texture analysis of the fresh plums are indicative of a tissue with well-packed parenchyma cells, bonded together by strong intercellular adhesion.

Boiling caused a remarkable decrease on firmness, rigidity, and deformation work of the fruits of both orchards. The firmness of the fruits (with skin) decreased an average of 87% for both orchards, while a decrease of 80% for VV and 90% for CA were observed in the firmness of the fruits flesh (without skin) with boiling (Fig. 4a and b). Rigidity showed an average decrease with the boiling step of 94% for VV and 98% for CA plums analysed with and without skin, relatively to fresh plums (Fig. 4c and d). The total work needed to the tissue rupture also decreased with boiling of plums (Fig. 4e and f), by values of 90% or higher. These results indicate that the heat treatment caused tissue softening, which is in accordance with the reduction of the strength of cellular adhesion and the increase of the intercellular spaces observed by SEM. The texture of boiled plums tissue is characteristic of that of a soft solid consisting of individual loosely bound cells. The easier cell separation has a very important role in the texture profile after processing, as was shown for dried pears (Ferreira et al., in press).

The candying process caused an increase in firmness, rigidity, and deformation work for plums from both orchards, compared to the boiled plums. The firmness of the fruits increased 3-folds for plums of both orchards, while rigidity increased 1.5-fold for VV and 3-fold for CA plums (Fig. 4a-d). The deformation work exhibited by the candied plums under puncture tests increased remarkably, reaching values similar to the fresh tissue (Fig. 4e and f). This result also supports the idea stated above that more energy would be required for

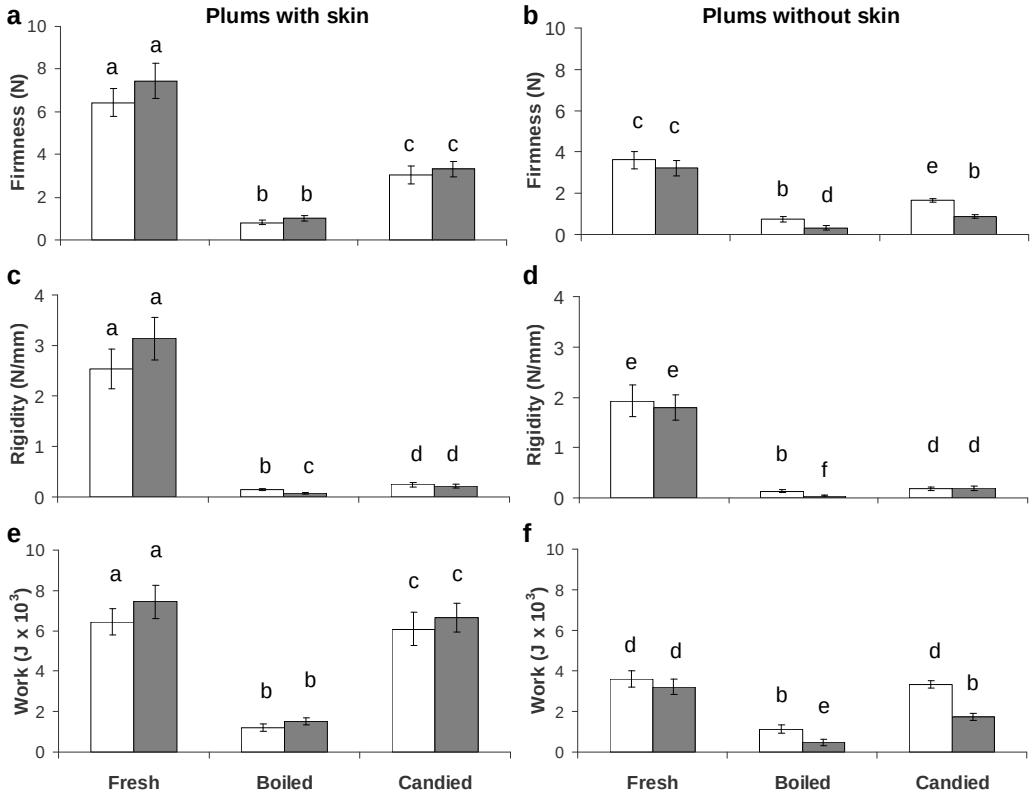

Fig. 4. Texture parameters obtained from puncture tests for Vila Viçosa (□) and Cano (■) plums, with and without skin, during processing (fresh, boiled, and candied). (a) and (b), firmness; (c) and (d), rigidity; (e) and (f), deformation work. For each parameter (firmness, rigidity, and deformation work), different letters represent values that are significantly ($p < 0.05$) different (n = 30).

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cell-to-cell adhesion in the candied parenchyma tissue. The presence of the skin caused an improvement of the texture parameters, particularly firmness and deformation work, also revealing some recovery of the skin tissue texture properties that were lost during the boiling step. The partial recovery of the texture properties of the fresh plums by the candied plums was in accordance to the partially restored shape and cell-to-cell adhesion of parenchymatous cells observed by SEM.

Between orchards, no significant variations were found in texture of the fresh plums. Boiled VV plums without skin showed a higher firmness, rigidity and deformation work when compared to CA. With skin, this was only visible concerning the rigidity. The main differences in the texture properties of the candied plums of the two orchards occurred when the puncture measurements were done without skin, with plums from the CA orchard showing equal rigidity but clearly smaller ($p < 0.05$) firmness and deformation work. This is probably the reason why candied CA plums show softening, resulting in a lower texture quality final product.

4. Concluding remarks

SEM and texture studies were carried out in plums from two orchards (CA and VV) during the candying process. The observation by SEM of the unprocessed plum tissue revealed that parenchyma was constituted by isodiametric and thickly packed cells, with smaller size cells near the epidermis of the fruits. Puncture analysis of the plums tissue revealed a texture profile characteristic of moderately hard fruits. After boiling, microstructure of plums from both orchards showed mainly cell separation and a huge decrease of the texture parameters. SEM images of candied plums evidenced tissue microstructure recovery, which was confirmed by the increase of firmness, rigidity, and deformation work of the plum tissue, for both orchards.

CA plums revealed to have lower values for the texture parameters analysed in the fruits without skin, confirming the lower texture quality of these candied plums. The conjugation of microstructure and texture analyses revealed that the recovery of texture properties of the candied plums was related to parenchyma cell structure, particularly the recuperation of middle lamella, incrementing cell-to-cell adhesion. Since pectic polysaccharides are the main constituents of primary cell walls and middle lamella, they might be involved on the recovery of the texture of candied plums. The osmotic dehydration and diffusion of sucrose into the fruits tissue and its interaction with the cell wall and middle lamella pectic polysaccharides might result in the formation of a jam-like structure that gives consistency to the tissues. Therefore, information on the cell wall polysaccharides composition in plums tissues and their interaction with sucrose, in a low water activity matrix, may provide a further understanding of the texture evolution of plums from the two studied orchards during processing. This will lead to establish the adequate stage of maturity and the properties of unprocessed fruits able to be used for candying, as well as the modulation of the processing parameters to avoid texture loss during the processing of ''Ameixa d'Elvas'' candied plums from CA orchard and from orchards that show the same problem.

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EFFECT OF CANDYING ON CELL WALL POLYSACCHARIDES OF PLUMS (*PRUNUS DOMESTICA* L.) AND INFLUENCE OF CELL WALL ENZYMES

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Effect of candying on cell wall polysaccharides of plums (*Prunus domestica* L.) and influence of cell wall enzymes

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ABSTRACT

"Ameixa d'Elvas" is a candied plum (*Prunus domestica* L.) produced by a traditional process, using fruits of a specific 'greengage' variety, ''Rainha Cláudia Verde". The candying process consists of boiling the intact plums in water for 15 min and then putting them in sugar syrup, which is successively concentrated until 75 ºBrix. Although a loss of intercellular adhesion of parenchyma cells after boiling is observed, candied plums are able to recover their cell-to-cell adhesion, giving a final tissue with a consistency similar to that observed for the fresh fruit. In order to explain this observation, cell wall polysaccharides of plums harvested in two orchards, Vila Viçosa (VV) and Cano (CA), from the same geographic region and at the same stage of ripening, were analysed fresh, boiled and candied. Plum cell walls are composed mainly of pectic polysaccharides and cellulose that, during the boiling step, are degraded and solubilised. Highly esterified pectic polysaccharides undergo gelation inside the fruits in the presence of sucrose, leading to the recovery of the fruit's consistency. During the candying process diffusion of these methylesterified pectic polysaccharides to the sucrose syrup increase the syrup viscosity. The activity of pectin methylesterase, polygalacturonase, and cellulose of fresh fruits explains the observed higher extension of degradation of cell wall polysaccharides of the CA plum tissues after boiling. This higher degradation seems to prevent the complete recovery of the parenchyma cell structure, which was observed for the less degraded polysaccharides of VV plums.

IX

1. Introduction

The relative amounts of cell wall polysaccharides, as well as the type, length, and branching pattern of their side-chains have a profound effect on the structure of the cell walls and, consequently, in the texture of fruits (Van Buren, 1979; Waldron, Smith, Parr, Ng, & Parker, 1997; Wakabayashi, 2000). Generally, fruit cell walls contain a large amount of pectic polysaccharides, being the major constituents of the middle lamella and thus contributing to the cell adhesion mechanism and cell packing (Harker, Stec, Hallett, & Bennett, 1997; Waldron et al., 1997). Hemicelluloses, like xyloglucans and cellulose, also play a significant structural function in the cell wall, thus influencing the textural properties of the fruits. Cellulose microfibrils and xyloglucans form a strong structure around each cell, which is embedded in a matrix of interconnected pectic polysaccharides (Vincken et al., 2003). These interactions between the semi-rigid cellulose microfibrils and the less rigid polysaccharide molecules also play a key role in the mechanical properties of the fruits. The cell wall polysaccharide composition is characteristic for each fruit and is important in understanding its texture. Concerning plums, as far as we know, no study is yet available on this subject.

Heat processing promotes softening of fruit tissue, resulting in the increase of middle lamella cell separation and/or wall weakening, due to the depolymerisation of the methylesterified pectic polysaccharides by a mechanism of β-eliminative degradation (Van Buren, 1979; Waldron et al., 1997), exhibiting lower average molecular weight. The proportion of neutral sugar side-chains is also an important factor contributing to textural changes during the heating process, since the side-chains of the pectic polysaccharides have been shown to interact with hemicelluloses, e.g., xyloglucans and cellulose (Prasanna, Prabha, & Tharanathan, 2007). The amount and the molecular mass of xyloglucans decrease with heat processing and a partial breakdown of the cellulose–xyloglucan network could occur, decreasing the integrity of the cell wall architecture. In addition, the solubilisation of hemicelluloses and cellulose have been also related to texture loss after heating (Mafra, Barros, & Coimbra, 2006a, 2007). A relevant technological aspect of cell structure modification of fruits caused by heat treatment is the increase of the flow rate diffusion of sucrose during fruits osmotic dehydration, as was observed for apples (Nieto, Salvatori, Castro, & Alzamora, 1998).

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The decline in cell wall strength and cell-to-cell adhesion resulting in tissue softening in fresh fruits is also associated with fruit ripening, where structural changes in all cell wall polysaccharides occur (Brummell, Dal Cin, Lurie, Crisosto, & Labavitch, 2004; Femenia, Sanchez, Simal, & Rossello, 1998; Mafra et al., 2006b; Wakabayashi, 2000). Pectin methylesterase (PME) catalyses the hydrolysis of methylester groups from galacturonosyl residues of pectic polysaccharides. PME play an important role in determining the extent to which demethylesterified pectic polysaccharides are accessible to degradation by polygalacturonases (PG) (Fischer & Bennett, 1991). High pectin demethylesterification, as catalysed by PME, also has the purpose of modifying the pH and the cation-exchange properties of the walls, which modulates the activity of other wall-degrading enzymes (Ali, Chin, & Lazan, 2004; Barnavon et al., 2001; Micheli, 2001). Cell wall loosening has also been explained by the disruption of non-covalent bonds between xyloglucan and cellulose (Cutillas-Iturralde, Pena, Zarra, & Lorences, 1998). The remarkably high levels of cellulase have been implicated in the change of cellulose fibril organisation and decrease of xyloglucan molecular weight, as shown in a large number of fruits. The partial breakdown of the cellulose–xyloglucan network decreases the integrity of the cell wall, which may increase the wall pore size and could also enhance the mobility of other hydrolases in cell walls (Mafra et al., 2006b; Prasanna et al., 2007; Rosli, Civello, & Martinez, 2004;. Wakabayashi, 2000; Yashoda, Prabha, & Tharanathan, 2005)

Plums (*Prunus domestica* L.) of a special type of 'greengage' variety, ''Rainha Cláudia Verde", from Alto Alentejo (South-East of Portugal) can be utilised to obtain a traditional candied plum, ''Ameixa d'Elvas", which has a protected designation of origin (PDO) recognised by the European Union. The candying process consists in boiling the intact plums in water for 15 min and then put them in sugar syrup, which is successively concentrated until 75 ºBrix. The main objective of this work is to study the cell wall polysaccharides of plums and to evaluate the changes that occur during candying. Also, analysis of cell wall degrading enzymes was performed on the fresh fruits to better evaluate their stage of maturity. In addition, analysis of polysaccharides was performed in the final sucrose syrup, to evaluate polysaccharide diffusion from the fruits to the liquid media.

2. Materials and methods

2.1. Plant material

Plums (*P. domestica* L.) of 'greengage' variety 'Rainha Cláudia Verde' were collected at the established stage of ripening suitable for candying (16–17 ºBrix, pH 3.3, and titratable acidity of 1 meq of malic acid per 100 g of fresh weight) in two orchards, Vila Viçosa (VV) and Cano (CA). The candying process was carried out by boiling the plums in water (water:fruit ratio of 4:1) for 15 min, followed by immersion of the intact boiled plums in 60 ºBrix sucrose syrup (syrup:fruit ratio of 2:1) for one day. The sucrose solution was then concentrated until 65 ºBrix and, after 7 days, until 75 ºBrix. The plums were stored two months in the 75 ºBrix sucrose syrup, its concentration being occasionally (2–3 times) corrected due to its hygroscopicity.

The analyses of cell wall polysaccharides were performed on fresh, boiled, and candied plums. The plums were collected in the factory (Confibor Lda; Estremoz, Portugal), brought immediately to the laboratory and were frozen with liquid nitrogen and maintained at -20 ºC until analysis. The sucrose syrup was used for the study of polysaccharides and was also maintained at -20 ºC until analysis.

2.2. Preparation of cell wall material

Plums (500 g) were destoned and the flesh was dispersed in ethanol (2 l) at a final concentration of 85% (v/v), and boiled for 10 min. The mixture was cooled and filtered through a glass fibre filter (Whatman GF/C). The residue was dispersed again in ethanol, boiled for 10 min, and filtered. The residue was then washed with diethyl ether and allowed to dry at room temperature. The dried material was named the alcohol insoluble residue (AIR).

2.3. Sequential extraction of AIR

The AIR was extracted according to the method described by Mafra et al. (2001). The AIR (8 g) was sequentially extracted with: (1) water, 600 ml for 16 h at 4 ºC; (2) water, 500 ml for 6 h at room temperature; (3) 0.5 M imidazole/HCl pH 7.0, 600 ml of solution for 16 h at room temperature; (4) 0.5 M imidazole/HCl pH 7.0, 500 ml of solution for 2 h at room temperature; (5) 50 mM Na_2CO_3 + 20 mM $NaBH_4$, 600 ml, for 16 h at 4 ºC; (6) 50 mM Na_2CO_3 + 20 mM $NaBH_4$, 500 ml, for 2 h at room temperature; (7) 0.5 M KOH + 20 mM $NaBH_4$, 500 ml, for 2 h at 4 ºC; (8) 1 M KOH + 20 mM $NaBH_4$, 500 ml, for 2 h at 4 ºC; (9) 1 M KOH + 20 mM $NaBH_4$, 500 ml, for 2 h at room temperature; (10) 4 M KOH + 20 mM NaBH4, 500 ml, for 2 h at room temperature; (11) 4 M KOH + 3.5% H_3BO_3 + 20 mM $NaBH_4$, 500 ml, for 2 h at room temperature; and (12) 8 M KOH + 20 mM $NaBH_4$, 500 ml, for 2 h at room temperature. The KOH extractions were carried out with O_2-free solutions under N_2. After each extraction, the solubilised polymers were separated from the insoluble residue by centrifugation (24,400*g* for 10 min at 4 ºC) followed by filtration of the supernatant through a glass fibre filter (Whatman GF/C). The Na_2CO_3 and KOH extracts were neutralised to pH 5–6, in the cold, with glacial acetic acid, prior to dialysis. The residue (cellulosic residue – CR) obtained after the alkali extractions was suspended in water, neutralised (pH 5–6) and dialysed. After dialysis, all extracts were concentrated under reduced pressure and precipitates were collected separately by centrifugation (24,400*g* for 10 min at 4 ºC). The supernatant from the dialysis of the CR was collected separately from the residue by centrifugation and filtration. All extracts were frozen and freeze-dried.

2.4. Carbohydrate analysis

Monosaccharides were released from cell wall polysaccharides by a pre-hydrolysis in 0.2 ml of 72% H_2SO_4 for 3 h at room temperature, followed by 2.5 h hydrolysis in 1 M H_2SO_4 at 100 ºC (Selvendran, March, & Ring, 1979). Neutral sugars were analysed after conversion to their alditol acetates by GC, using 2-deoxyglucose as internal standard (Coimbra, Delgadillo, Waldron, & Selvendran, 1996) and GC analysis as described by Nunes, Rocha, Saraiva, and Coimbra (2006). Cellulosic glucose was calculated as the difference between the content found with and without 72% H_2SO_4 pre-hydrolysis.

Uronic acids (UA) were quantified by a modification (Coimbra et al., 1996) of the 3-phenylphenol colorimetric method (Blumenkrantz & Asboe-Hansen, 1973). Samples were prepared by hydrolysis in 0.2 ml of 72% H_2SO_4 for 3 h at room temperature followed by 1 h in 1 M H_2SO_4 at 100 ºC. A calibration curve was made with D-galacturonic acid.

The hydrolysis of all samples was done in duplicate and each one was injected twice. A third analysis was done for the few samples with higher variability.

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2.5. Determination of the degree of methylesterification and acetylation

The determination of the degree of methylesterification and acetylation of the pectic polysaccharides was based on the estimate of methanol and acetic acid contents released after saponification (Waldron & Selvendran, 1990), as described previously by Nunes, Rocha et al. (2006). The methanol and acetic acid were extracted from the headspace of the HCl acidified (pH 2) aqueous solution by solid phase microextraction (HS-SPME), using a DVB /Carboxen/PDMS fibre. The analytes were separated by gas chromatography and detected using a flame ionisation detector (GC-FID). External calibration curves were used. All measurements were made with at least three replicates. Blanks were run in between each set of experiments. The degree of methylesterification was calculated in relation to mol% of uronic acids and the acetylation in relation to mol% of the total sugars determined for the extracts analysed.

2.6. Quantification of enzymatic activity

The extraction of plum enzymes was performed according to a slightly modified method described by Denès, Baron, and Drilleau (2000), to prevent loss of activity by phenolic inhibition. The flesh of plums (100 g) was homogenised with the addition of 100 ml 0.2 M Tris(hydroxymethyl)-aminomethane (Tris) buffer, at pH 7, containing 500 mg/l sodium metabisulfite ($Na_2S_2O_5$) and 1% PVPP (polyvinylpolypyrrolidone). After extraction (2 h at 4 ºC), the suspension was centrifuged at 20,000*g* for 15 min at 4 ºC and the supernatant obtained was used as the source of soluble fraction enzymatic extract. The pellet was dispersed in the same buffer, but containing 1 M NaCl, and stirred for 2 h at 4 ºC, followed by centrifugation (20,000*g* for 15 min). The supernatant obtained was used as the source of cell wall ionically-linked fraction enzymatic extract. The pellet was used to determine the activity of the enzymes strongly linked to the cell wall, which were called cell wall covalently-linked enzymatic fraction.

Enzymatic activity quantification was determined in triplicate and expressed on plum fresh weight basis, for all enzymes. Total groups per min, under the previously mentioned assay conditions.

2.6.1. Pectin methylesterase (PME)

PME activity was measured by continuous recording of titration of carboxyl groups released from a pectin solution using an automatic pH-stat (Crison micro TT2022, Alella, Spain) and a 0.01 M NaOH solution (Nunes et al., 2006). Assays were performed with a 3.5 mg/ml apple pectin solution (degree of esterification 75%, 30 ml) containing 0.117 M NaCl at pH 7.0 and 25 ºC. The quantification of activity was performed adding, to 15 ml of pectin solution, 0.5 ml of enzyme extract, for soluble and ionic extracts, or approximately 1 g solid residue, for the covalent fraction. The blank assays were performed by adding each enzymatic extract to 15 ml of water. The value obtained for the possible formation of carboxyl groups was subtracted from the previously measured enzyme activity. One unit (U) of PME activity was defined as the amount of enzyme necessary to generate 1 μmol of carboxyl groups per min, under the previously mentioned assay conditions.

2.6.2. Polygalacturonase (PG)

The quantification of PG activity was based on the method described by Gross (1982). The method consists of the measurement of the released reducing groups from polygalacturonic acid. The substrate solution contained 0.4% (w/v) polygalacturonic acid in 0.05 M sodium acetate buffer (pH 4.5). The reaction was carried out by adding 0.2 ml of enzyme extract (soluble and ionic fractions) or 0.1 g for covalent fraction, and incubating at 35 ºC for 10 min. The reaction was stopped with 2 ml of 10 mM borate buffer at pH 9 and 0.4 ml of 1% (w/v) 2-cyanoacetamide. The blank assay was done adding the enzymatic extracts only, after the addition of borate buffer and 2-cyanoacetamide. The mixture was put in a boiled water bath for 10 min and after cooling the absorbance at 276 nm was measured. The amount of released reducing groups was determined by comparing with a calibration curve made with D-galacturonic acid, and the enzyme activity was expressed as nmol of galacturonic acid released per min.

2.6.3. Cellulase (Cel)

The method of quantification of Cel activity consists on the measurement of the solution viscosity, using a Cannon–Fenske capillary viscometer (75 mm), of the substrate solution before and after the action of the enzyme during 60 min at 30 ºC. The substrate solution consisted of 0.1% (w/v) carboxymethylcellulose in 0.1 M acetate buffer at pH 4 (Lohani, Trivedi, & Nath, 2004). In a standard assay 0.50 ml of acetate buffer, 0.50 ml of adequately diluted enzymatic extract (soluble and ionic fractions), a proper amount of solid residue (covalent fraction) and 0.50 ml more buffer were added to 4 ml of substrate solution. One Cel unit (U) was defined as 1% viscosity reduction per min.

2.6.4. Viscosimetric analysis

The viscosity of the sugar syrups was determined using shear stress measurements, at 25 ºC, with a TA AR-1000 rheometer (TA Instruments Ltd., Crawley, UK) fitted with a cone-plate device (4 cm and 6 cm, 2º).

2.7. Statistical analysis

Results are presented as mean value and the reproducibility of the results was expressed as standard deviation in tables and as error bars in figures. Statistical analysis between experimental results was based on Student's *t*-test. Significant difference was statistically considered at the level of $p < 0.05$.

3. Results and discussion

Cell wall polysaccharides composition of plums and the changes that occur during candying were studied. The fruits collected in two orchards, Vila Viçosa (VV) and Cano (CA), from the same geographic region within the PDO zone, and at the same stage of ripening, were analysed in fresh plums, after boiling, and after candying.

3.1. Cell wall material composition

Table 1 shows the physical characteristics of the fruits and the yield and sugars composition of the alcohol insoluble residues (AIR) prepared using fruits from two orchards, VV and CA. The fresh plums from VV had an average weight of 42 g and a volume of 34 ml/fruit, whereas those of CA were, on average, 33% lighter and 26% smaller. After the boiling process, the fruits increased their weight and volume by about 10–15%, due to the increase in their water content. However, the candied samples from the two orchards showed different weight and volume change tendencies with processing. In relation to the fresh fruits, those from VV decreased in weight (6%) and volume (12%), whereas those from CA increased in weight (14%) and recovered the volume measured for the fresh fruits. These results may be explained by the balance between the loss of water (75%) and the diffusion of sucrose into the flesh, resulting in a final product with only 22–23% of water. This balance was negative for bigger fruits and positive for the smaller ones.

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Table 1
Physical characteristics and sugar composition of AIR extracts of fresh, boiled, and candied plums from Vila Viçosa (VV) and Cano (CA) orchards

Orchard	Fresh		Boiled		Candied	
	VV	CA	VV	CA	VV	CA
Fruit weight (g)	41.9 ± 0.2[a]	27.7 ± 1.3[b]	47.2 ± 1.7[c]	30.2 ± 2.9[b,d]	39.2 ± 0.2[e]	32.3 ± 0.7[d]
Volume (ml)	34 ± 1	25 ± 1	38 ± 2	29 ± 1	30 ± 2	25 ± 1
Moisture (%)	84 ± 1	85 ± 0	88 ± 0	92 ± 1	22 ± 1	23 ± 0
AIR Yield [1] (g/kg)	33	33	37	41	15	19
AIR Yield [2] (mg/fruit)	749	658	617	387	247	399
AIR polysaccharides (mg/fruit)	338	417	241	223	144	192
AIR sugars (mg/fruit)						
Rha	7 ± 1	4 ± 0	5 ± 1	4 ± 0	3 ± 0	4 ± 0
Fuc	5 ± 0	3 ± 1	-	3 ± 0	2 ± 0	3 ± 0
Ara	56 ± 2	60 ± 1	33 ± 1	33 ± 2	25 ± 3	32 ± 1
Xyl	14 ± 1	13 ± 0	11 ± 1	11 ± 1	9 ± 0	11 ± 0
Man	9 ± 1	6 ± 1	5 ± 1	5 ± 1	3 ± 0	5 ± 0
Gal	58 ± 5	58 ± 1	43 ± 2	39 ± 1	29 ± 2	33 ± 1
Glc	43 ± 21	87 ± 1	41 ± 5	29 ± 1	14 ± 0	21 ± 1
UA	147 ± 11	185 ± 20	103 ± 10	100 ± 7	58 ± 5	82 ± 3

Mean ± standard deviation (n = 4).
Means with the same superscript letter are not significantly ($p > 0.05$) different.
[1] Yield is expressed in g of dry weight material per kg of fresh weight plum.
[2] Yield is expressed in mg of dry weight material per plum.

The AIR was used to obtain the polymeric material. On a fresh weight basis of the flesh, the AIR represented 3.3%, 3.7–4.1%, and 1.5–1.9% of fresh, boiled, and candied fruits. These values show that the amount of polymeric material present in the flesh of the plums in the different stages of processing is, apparently, independent of the size of the fruit. However, on a fruit basis, the AIR yield was only slightly higher in VV (0.75 g) than in CA (0.66 g), which suggests a relatively higher cell wall content in the smaller fruits. After boiling, the content of AIR in VV decreased 18%, whereas a higher decrease in the content of AIR (41%) was observed in CA. This result suggests a higher degradation and/or solubilisation of this polymeric material in aqueous media. The lowest AIR yields were obtained for the candied fruits. In these samples, the amount of AIR in VV was 0.25 g, decreasing its amount 67% in relation to the amount present in the fresh fruits, whereas the amount of AIR in CA was 0.40 g, decreasing its amount 40% in relation to the amount present in the fresh fruits and maintaining its amount in comparison to the boiled ones. These results show a larger loss of polymeric material from fresh to candied plums in VV (0.50 g/fruit) than in CA (0.26 g). According to the sugars analysis of the AIR (Table 1), the polysaccharides represent, in all samples, nearly 60% of the AIR. As a consequence, the relative loss of polysaccharides during processing was similar to that observed for the AIR: 0.50 and 0.26 g/fruit, in VV and CA, representing a 60% and 54% decrease, respectively, from fresh to candied fruits. The diffusing of the polymeric material to the aqueous media seems to explain its lower amount in the final product. This is in accordance with previous observations showing that, during osmotic dehydration of fruits in sucrose solutions, a two-way mass transfer is established. Water and water-soluble substances (sugars, vitamins, pigments, organic acids, mineral salts, etc.) flow out of the fruit into the solution, while in the opposite direction sucrose is transferred from the solution to the fruits (Giraldo, Talens, Fito, & Chiralt, 2003; Peiró, Dias, Camacho, & Martinez-Navarrete, 2006).

Uronic acids are the most abundant sugars of the cell wall polysaccharides of the plums. Also abundant are arabinose, galactose, and glucose. This sugar composition implies that pectic polysaccharides, composed of uronic acid, Ara and Gal, together with cellulose, are abundant in plums. All of these sugars decrease their amount per fruit during the processing, showing the same tendency observed for the amount of AIR and polysaccharides, that is, a higher decrease with boiling in CA, whereas in VV the higher decrease is observed from boiled to candied fruits. In order to observe the composition of the cell wall polysaccharides with processing, and explain the differences in the sugars composition of the AIR, this material was submitted to a sequential extraction with aqueous solvents and the sugars present in all extracts were analysed. The results are shown in Tables 2–4 for the fresh, boiled, and candied plums, respectively.

3.2. Pectic polysaccharides

The extracts rich in pectic polysaccharides are recovered with water, with aqueous solutions of chelating agents, and with solutions of dilute carbonate (Coimbra et al., 1996). Also, it has been shown that in the supernatant of the dialysis of the cellulosic residue obtained after neutralisation (snCR) is a fraction mainly composed of pectic polysaccharides (Ferreira, Mafra, Soares, Evtuguin, & Coimbra, 2006).

The extraction of the AIR with water and imidazole allowed the extraction of 7.4–9.3% of polymeric material from fresh, 9.5–9.8% from the boiled, and 22.3–27.1% from the candied plums. These extracts were very rich in polysaccharides in all samples (63–90%), and the main sugars were those characteristic of pectic polysaccharides. The ratio Ara/Gal was nearly 1 in all these pectic polysaccharide samples. However, the ratio of the amount of uronic acid to the amount of Ara + Gal changed from 4 in fresh to 2 in boiled and candied plums, showing that the pectic polysaccharides recovered with water and imidazole from boiled and candied fruits were more branched than those recovered in these extracts from fresh plums. The UA/(Ara + Gal) ratio was 8 in fresh plums in the extracts obtained from the dilute sodium carbonate solutions at 4 ºC, and 2 in the extracts obtained at 22 ºC. As observed for the water and imidazole extracts, in these carbonate-extracted pectic polysaccharides, the UA/(Ara + Gal) ratio also decreased. In boiled samples, the ratio was 5 and 1, for the carbonate extracts obtained at 4 ºC and 22 ºC, respectively, and 2 and 0.5 in candied samples. In the snCR fraction, the recovered pectic polysaccharides had an UA/(Ara + Gal) ratio of 0.5 in all samples. The relatively high proportion of Ara and Gal in the final residue, as well as the presence of UA, suggests the occurrence of a fraction of highly-branched pectic polysaccharides that were not recovered from the polymeric matrix with the solvents used. This was more relevant in the fresh fruits than in the processed ones. The increase of branched pectic polysaccharides with heat processing was also observed for other fruits and vegetables, which is related with the loss of firmness

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Table 2
Sugar composition of fractions of fresh plum AIR obtained by sequential extraction with aqueous solvents for Vila Vicçosa (VV) and Cano (CA) orchards

Extract	Orchard	Yield[a] (%)	Cell wall sugars (mol%)								Total (mg/g)
			Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA	
Water	VV	4.3	2 ± 0	1 ± 0	18 ± 1	5 ± 0	3 ± 0	17 ± 1	6 ± 1	48 ± 2	783
	CA	5.7	1 ± 0	1 ± 0	16 ± 1	4 ± 0	2 ± 0	14 ± 0	10 ± 0	51 ± 1	767
Imidazole	VV	2.1	2 ± 0	1 ± 0	11 ± 1	3 ± 0	3 ± 0	12 ± 0	5 ± 0	64 ± 1	735
	CA	3.6	1 ± 0	tr	10 ± 0	2 ± 0	1 ± 0	7 ± 0	2 ± 0	76 ± 1	806
Na_2CO_3 4 ºC	VV	8.0	2 ± 0	–	7 ± 1	1 ± 0	–	4 ± 1	tr	85 ± 1	483
	CA	8.5	1 ± 0	–	8 ± 0	tr	–	3 ± 0	–	87 ± 1	794
Na_2CO_3 20 ºC	VV	1.7	2 ± 0	–	22 ± 2	1 ± 0	–	16 ± 1	1 ± 0	59 ± 4	466
	CA	2.3	2 ± 0	–	24 ± 1	1 ± 0	–	13 ± 1	–	61 ± 0	619
0.5 M KOH sn	VV	0.7	2 ± 0	2 ± 0	13 ± 1	33 ± 1	5 ± 0	13 ± 1	13 ± 1	18 ± 1	451
	CA	1.3	4 ± 0	–	5 ± 0	34 ± 1	5 ± 0	10 ± 1	24 ± 0	18 ± 0	786
0.5 M KOH pp	VV	2.0	2 ± 0	3 ± 0	29 ± 1	9 ± 0	1 ± 0	27 ± 0	12 ± 0	16 ± 1	326
	CA	2.0	1 ± 0	6 ± 0	14 ± 0	22 ± 0	1 ± 0	17 ± 0	26 ± 0	13 ± 0	387
1 M KOH sn	VV	4.0	2 ± 0	3 ± 0	23 ± 1	15 ± 1	3 ± 0	22 ± 2	12 ± 0	20 ± 5	561
	CA	5.0	2 ± 0	2 ± 0	35 ± 0	11 ± 0	1 ± 0	28 ± 0	5 ± 0	16 ± 0	549
1 M KOH pp	VV	0.3	3 ± 0	2 ± 0	33 ± 0	10 ± 1	3 ± 0	23 ± 1	11 ± 0	15 ± 1	265
	CA	0.3	2 ± 0	1 ± 0	39 ± 2	9 ± 0	1 ± 0	26 ± 0	10 ± 2	11 ± 0	235
4 M KOH sn	VV	3.4	2 ± 0	2 ± 0	26 ± 0	11 ± 0	10 ± 0	20 ± 0	16 ± 0	12 ± 1	555
	CA	4.8	2 ± 0	2 ± 0	28 ± 0	11 ± 0	7 ± 0	26 ± 0	13 ± 0	11 ± 0	612
4 M KOH pp	VV	0.5	3 ± 0	2 ± 0	36 ± 3	15 ± 1	5 ± 0	14 ± 0	14 ± 2	12 ± 1	234
	CA	0.3	1 ± 0	1 ± 0	42 ± 0	16 ± 0	2 ± 0	12 ± 1	6 ± 0	21 ± 1	437
8 M KOH sn	VV	0.5	2 ± 0	2 ± 0	27 ± 0	9 ± 1	8 ± 1	22 ± 1	16 ± 1	13 ± 3	700
	CA	2.6	3 ± 0	tr	37 ± 0	2 ± 0	–	29 ± 0	1 ± 0	28 ± 0	770
8 M KOH pp	VV	0.4	3 ± 0	–	52 ± 1	10 ± 0	2 ± 0	14 ± 1	7 ± 0	13 ± 1	265
	CA	0.8	2 ± 0	–	40 ± 1	2 ± 0	–	30 ± 0	tr	25 ± 1	637
snCR	VV	6.5	2 ± 0	–	32 ± 1	1 ± 0	–	31 ± 1	–	35 ± 1	958
	CA	1.2	2 ± 0	–	32 ± 1	1 ± 0	–	23 ± 1	–	42 ± 0	767
CR	VV	38.8	3 ± 1	–	28 ± 4	4 ± 1	1 ± 0	18 ± 1	24 ± 9	22 ± 5	303
	CA	14.0	2 ± 0	–	19 ± 0	2 ± 0	1 ± 0	13 ± 0	39 ± 0	25 ± 0	848

Mean ± standard deviation (n = 4).
tr, trace amount.
sn, material soluble in water recovered after neutralisation and dialysis of the extract.
pp, material insoluble in water recovered after neutralisation and dialysis of the extract.
CR, cellulosic residue.
[a] Yield is expressed in mg of dry weight material per 100 g of AIR.

of the tissues (Hurtado, Greve, & Labavitch, 2002; Ratnayake, Melton, & Hurst, 2003; Stolle-Smits, Beekhuizen, Recourt, Voragen, & Van Dijk, 1997).

On a fruit basis, the amount of uronic acids, Ara, and Gal decreased with boiling and candying, although different behaviours were found for the fruits from the two orchards (Fig. 1). The fresh fruits of VV contained less uronic acids than CA in all combined extracts, except in snCR (Fig. 1a). In these snCR extracts, the amount of uronic acids of fresh plums from VV reached 20 mg/fruit whereas in CA it was only 4 mg/fruit. Lower values of uronic acids were also found in all boiled and candied fruits (1–4 mg/fruit). These results allow us to infer that a large amount of pectic polysaccharides of fresh VV plums were entrapped in the hemicellulosic and cellulosic matrix. In strong alkali, the ionisation of the $–CH_2OH$ groups on cellulose could prevent the diffusion of negatively-charged pectic polysaccharides enmeshed within the swollen cellulose matrix. Upon neutralisation of the cellulose-rich suspension, the loss of negative charges on cellulose facilitates diffusion of the entangled pectic polysaccharides (Coimbra, Waldron, & Selvendran, 1994). In all other plum samples, the previous degradation of pectic polysaccharides and/or the swelling of the cellulosic matrix could have allowed their solubilisation by the water, imidazole, and carbonate extracts. Fig. 1b shows that the amount of Ara + Gal of fresh plums is more evenly distributed than uronic acids through the combined pectic extracts (19 and 26 mg/fruit in VV and CA, respectively), KOH extracts (22 and 45 mg/fruit), snCR (31 and 4 mg/fruit), and cellulosic residue (42 and 37 mg/fruit). The exception was the snCR of CA, as already stated for the uronic acids. The fresh, boiled, and candied fruits were shown to have the same amount of Ara + Gal recovered by the pectic extracts. However, these two sugars, which mostly arise from the pectic polysaccharide side-chains, decreased their content on a fruit basis (70–80%) mainly in the cellulosic residue. The solubilisation observed for the uronic acids in the pectic extracts in boiled and candied plums was also observed in the Ara + Gal, which was more pronounced in the candied than in the boiled samples. These results show that an important amount of pectic polysaccharides, although degraded, was still present in the cell wall matrix and diffused during the osmotic process. The higher diffusion of UA-rich polymers during candying observed for VV than for CA is in accordance with the already reported loss of these polymers during the boiling process.

3.3. Degree of methylesterification and activity of cell wall pectic enzymes

The esterification with methanol or acetic acid is a very important structural characteristic of pectic polysaccharides, since it could be related with the change in texture of the fruits during processing. In order to evaluate if the different characteristics observed on cell wall pectic polysaccharides between the two orchards could be related to the methylesterification or acetylation

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Table 3
Sugar composition of fractions of boiled plums AIR obtained by sequential extraction with aqueous solvents for Vila Viçosa (VV) and Cano (CA) orchards

Extract	Orchard	Yield[a] (%)	Cell wall sugars (mol%)								Total (mg/g)
			Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA	
Water	VV	7.1	2 ± 0	1 ± 0	13 ± 1	3 ± 0	2 ± 0	11 ± 1	10 ± 1	57 ± 3	727
	CA	7.5	2 ± 0	1 ± 0	10 ± 0	3 ± 0	2 ± 0	11 ± 0	6 ± 1	66 ± 2	886
Imidazole	VV	2.4	2 ± 0	1 ± 0	18 ± 1	2 ± 0	2 ± 0	11 ± 1	3 ± 0	61 ± 2	753
	CA	2.3	1 ± 0	1 ± 0	25 ± 2	2 ± 0	2 ± 0	8 ± 0	2 ± 0	60 ± 2	902
Na_2CO_3 4 ºC	VV	6.5	1 ± 0	tr	6 ± 0	1 ± 0	tr	6 ± 0	tr	85 ± 1	926
	CA	17.5	2 ± 0	1 ± 0	19 ± 1	1 ± 0	–	16 ± 1	–	62 ± 1	716
Na_2CO_3 20 ºC	VV	2.2	3 ± 0	1 ± 0	26 ± 1	2 ± 0	1 ± 0	22 ± 1	1 ± 0	45 ± 2	573
	CA	4.6	1 ± 0	tr	27 ± 1	1 ± 0	–	26 ± 1	tr	44 ± 2	874
0.5 M KOH sn	VV	0.7	2 ± 0	4 ± 0	10 ± 1	29 ± 0	3 ± 0	16 ± 1	23 ± 2	14 ± 2	822
	CA	0.9	2 ± 0	5 ± 0	7 ± 0	34 ± 2	3 ± 0	12 ± 0	12 ± 0	27 ± 3	953
0.5 M KOH pp	VV	2.8	3 ± 0	2 ± 0	36 ± 1	4 ± 1	–	46 ± 1	2 ± 0	7 ± 1	225
	CA	1.3	4 ± 0	2 ± 0	24 ± 1	4 ± 0	2 ± 0	20 ± 1	4 ± 0	41 ± 2	197
1 M KOH sn	VV	2.6	2 ± 0	3 ± 0	19 ± 1	16 ± 1	3 ± 0	28 ± 1	17 ± 1	11 ± 1	826
	CA	1.3	2 ± 0	5 ± 0	5 ± 0	27 ± 1	6 ± 1	15 ± 1	20 ± 0	21 ± 2	845
1 M KOH pp	VV	1.2	2 ± 0	3 ± 1	31 ± 1	12 ± 1	2 ± 0	31 ± 2	5 ± 1	15 ± 1	428
	CA	1.8	4 ± 0	2 ± 0	24 ± 1	18 ± 2	2 ± 0	12 ± 1	3 ± 0	35 ± 2	197
4 M KOH sn	VV	1.9	3 ± 0	2 ± 0	23 ± 1	7 ± 1	7 ± 0	28 ± 1	13 ± 1	17 ± 1	971
	CA	3.6	2 ± 0	3 ± 0	18 ± 1	11 ± 1	7 ± 0	22 ± 1	13 ± 1	26 ± 3	655
4 M KOH pp	VV	0.4	3 ± 1	3 ± 1	36 ± 1	17 ± 0	3 ± 0	17 ± 1	9 ± 0	12 ± 2	213
	CA	0.8	3 ± 0	2 ± 0	18 ± 2	16 ± 1	3 ± 0	14 ± 1	12 ± 1	32 ± 2	560
8 M KOH sn	VV	0.2	3 ± 0	2 ± 0	28 ± 1	7 ± 0	9 ± 0	31 ± 0	17 ± 1	4 ± 0	588
	CA	1.2	2 ± 0	2 ± 0	34 ± 1	6 ± 0	2 ± 0	31 ± 0	6 ± 1	16 ± 1	871
8 M KOH pp	VV	0.3	2 ± 0	1 ± 0	37 ± 1	6 ± 0	1 ± 0	25 ± 0	4 ± 1	25 ± 1	614
	CA	0.2	3 ± 0	2 ± 0	21 ± 1	33 ± 2	2 ± 0	12 ± 0	6 ± 0	22 ± 2	405
snCR	VV	1.7	1 ± 0	tr	31 ± 1	1 ± 0	tr	28 ± 1	4 ± 0	34 ± 1	940
	CA	7.5	–	–	34 ± 2	–	–	26 ± 3	–	40 ± 5	450
CR	VV	14.6	2 ± 0	1 ± 0	19 ± 0	3 ± 0	1 ± 0	16 ± 0	43 ± 1	15 ± 2	544
	CA	9.8	2 ± 0	1 ± 0	15 ± 1	5 ± 0	2 ± 0	12 ± 0	35 ± 2	27 ± 1	494

tr, trace amount.
Mean ± standard deviation (n = 4).
sn, material soluble in water recovered after neutralisation and dialysis of the extract.
pp, material insoluble in water recovered after neutralisation and dialysis of the extract.
CR, cellulosic residue.
[a] Yield is expressed in mg of dry weight material per 100 g of AIR.

of pectic polysaccharides, the degrees of methylesterification and acetylation of the AIR and pectic polysaccharide-rich extracts not submitted to the alkali reagents were evaluated. Also, the activities of the enzymes pectin methylesterase (PME) and polygalacturonase (PG) were quantified in fresh plums from VV and CA orchards, since the activity of these enzymes can decrease the degree of methylesterification (DM) and the degree of polymerisation of pectic polysaccharides.

The DM, as well as the degree of acetylation (DA), determined for AIR, water, and imidazole extracts of fresh, boiled, and candied VV and CA plums are presented in Table 5. The DM of the pectic polysaccharides of AIR of VV fresh plums (57%) was higher than that of CA (38%). In the boiled plums, no statistical differences were observed, when compared to the values obtained for the fresh plums. However, the candying process caused a decrease of the DM of the pectic polysaccharides in the AIR to 44% and 26% for VV and CA plums, respectively.

The DM of the pectic polysaccharides present in the water extract of fresh plums of both orchards is similar to the DM observed for the AIR, which are lower than those determined for the imidazole extracts (65% and 75%, respectively, for VV and CA). After boiling, the DMs of the water extracts were significantly higher than those observed for the fresh plums, whereas the DMs of the imidazole extracts were significantly lower. The DM of the pectic polysaccharides present in the imidazole extract of boiled plums of both orchards is similar to the DM observed for the respective AIR. For the candied fruits, the DM of the pectic polysaccharides present in both water and imidazole extracts, of both orchards, is higher than the DM observed for their respective AIR. For VV plums, where no loss of pectic polysaccharides was observed between fresh and boiled fruits (Fig. 1a), these results indicate that the highly esterified pectic polysaccharides become more soluble with the heating treatment. During osmotic treatment some loss of these polysaccharides occurred, which is in accordance with the degradation of highly methylated regions of pectic polysaccharides by β-elimination due to heating (Stolle-Smits et al., 1997). The same process might have occurred in CA, although the results for this sample were not conclusive, due to the solubilisation of the pectic polysaccharides in the boiling step (Fig. 1a).

AIR and water extracts for fresh plums showed a DA between 5% and 7%, while for boiled and candied plums the percentage of acetyl groups per sugar residues tended to increase, reaching up to 15% in the water extracts of boiled plums from the two orchards (Table 5). The increase of the content of acetyl groups in pectic polysaccharides has been shown to prevent their gelling abilities (Renard & Jarvis, 1999). However, as in candied plums, the cell-to-cell adhesion of cells structure is recovered. It is possible that the high methylesterification of the pectic polysaccharides is an important factor for the recovery of the texture of the fruits after osmotic treatment. Boiled VV plums revealed a higher quantity of highly esterified (DM = 83%) soluble pectic polysaccharides, when

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Table 4
Sugar composition of fractions of candied plums AIR obtained by sequential extraction with aqueous solvents for Vila Viçosa (VV) and Cano (CA) orchards

Extract	Orchard	Yield[a] (%)	Cell wall sugars (mol%)								Total (mg/g)
			Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA	
Water	VV	22.7	2 ± 0	1 ± 0	12 ± 0	4 ± 0	2 ± 0	15 ± 0	13 ± 0	50 ± 1	882
	CA	19.1	2 ± 0	1 ± 0	13 ± 1	4 ± 0	2 ± 0	12 ± 1	12 ± 1	54 ± 2	781
Imidazole	VV	4.4	2 ± 0	1 ± 0	20 ± 1	2 ± 0	2 ± 0	13 ± 1	3 ± 0	57 ± 1	781
	CA	3.2	2 ± 0	1 ± 0	14 ± 1	3 ± 0	2 ± 0	16 ± 1	3 ± 0	58 ± 1	630
Na_2CO_3 4 ºC	VV	6.0	2 ± 0	1 ± 0	13 ± 0	1 ± 0	tr	14 ± 1	tr	69 ± 1	886
	CA	12.2	2 ± 0	1 ± 0	14 ± 1	1 ± 0	1 ± 0	15 ± 1	1 ± 0	66 ± 2	682
Na_2CO_3 20 ºC	VV	3.3	2 ± 0	–	29 ± 1	1 ± 0	–	34 ± 1	1 ± 0	33 ± 1	658
	CA	5.0	3 ± 0	1 ± 0	33 ± 1	2 ± 0	–	34 ± 2	1 ± 0	27 ± 1	484
0.5 M KOH sn	VV	3.9	3 ± 0	4 ± 0	14 ± 0	20 ± 0	2 ± 0	18 ± 1	15 ± 1	23 ± 2	300
	CA	1.0	1 ± 0	4 ± 0	7 ± 0	36 ± 1	4 ± 0	14 ± 1	19 ± 1	15 ± 2	817
0.5 M KOH pp	VV	1.2	4 ± 0	2 ± 0	23 ± 1	7 ± 0	3 ± 0	25 ± 1	16 ± 2	21 ± 1	214
	CA	1.2	2 ± 0	2 ± 0	32 ± 1	6 ± 0	1 ± 0	31 ± 1	5 ± 1	20 ± 2	278
1 M KOH sn	VV	3.9	2 ± 0	4 ± 0	13 ± 1	21 ± 1	5 ± 0	20 ± 2	18 ± 1	18 ± 2	695
	CA	5.1	2 ± 0	3 ± 0	15 ± 1	21 ± 1	6 ± 0	21 ± 1	14 ± 1	18 ± 1	713
1 M KOH pp	VV	1.9	5 ± 0	1 ± 0	43 ± 2	8 ± 0	1 ± 0	26 ± 1	6 ± 1	12 ± 0	190
	CA	3.3	3 ± 0	3 ± 0	44 ± 2	8 ± 0	1 ± 0	30 ± 2	5 ± 0	7 ± 0	267
4 M KOH sn	VV	4.8	2 ± 0	3 ± 0	16 ± 1	14 ± 1	10 ± 1	22 ± 1	23 ± 1	10 ± 1	735
	CA	6.9	3 ± 0	3 ± 0	20 ± 1	11 ± 0	5 ± 0	24 ± 1	13 ± 1	22 ± 1	544
4 M KOH pp	VV	2.6	3 ± 0	3 ± 0	43 ± 2	23 ± 2	3 ± 1	10 ± 1	6 ± 0	9 ± 1	178
	CA	3.4	3 ± 0	2 ± 0	32 ± 1	6 ± 1	2 ± 0	26 ± 2	6 ± 0	23 ± 2	376
8 M KOH sn	VV	0.7	2 ± 0	1 ± 0	34 ± 3	5 ± 0	4 ± 1	31 ± 1	8 ± 1	15 ± 2	937
	CA	2.5	3 ± 0	1 ± 0	31 ± 1	5 ± 0	3 ± 0	28 ± 2	5 ± 0	25 ± 2	535
8 M KOH pp	VV	0.4	5 ± 0	–	55 ± 1	20 ± 0	–	10 ± 0	7 ± 1	3 ± 0	122
	CA	0.6	3 ± 0	1 ± 0	31 ± 1	10 ± 0	2 ± 0	30 ± 1	8 ± 0	13 ± 1	513
snCR	VV	2.3	2 ± 0	1 ± 0	23 ± 2	1 ± 0	tr	23 ± 3	3 ± 0	47 ± 5	931
	CA	1.9	2 ± 0	–	33 ± 1	1 ± 0	–	37 ± 1	tr	26 ± 0	678
CR	VV	37.7	2 ± 0	1 ± 0	19 ± 1	4 ± 0	1 ± 0	19 ± 0	38 ± 2	16 ± 1	564
	CA	21.7	2 ± 0	1 ± 0	22 ± 1	4 ± 0	1 ± 0	20 ± 0	22 ± 0	27 ± 2	484

tr, trace amount.
Mean ± standard deviation (n = 4).
sn, material soluble in water recovered after neutralisation and dialysis of the extract.
pp, material insoluble in water recovered after neutralisation and dialysis of the extract.
CR, cellulosic residue.
[a] Yield is expressed in mg of dry weight material per 100 g of AIR.

compared to CA plums, which can explain the differences observed on the texture and microstructure of plums from the two orchards, where VV plums showed a higher recovery of cell size and shape and of cell-to-cell adhesion (Nunes, Pinto, Santos, Lopes da Silva, Saraiva, & Coimbra, 2008).

In order to evaluate the involvement of pectin methylesterase (PME) and polygalacturonase (PG) in these properties, the activity of these enzymes in the fresh fruits was evaluated. According to Table 6, PME activity was 28% higher in VV than in CA whereas PG activity was 6-fold higher in CA than in VV. Usually, the activity of PME in fruits increases with ripening, up to a point where it decreases in a concerted action with the increase of PG activity. The activity of PME renders pectins more susceptible to the action of PG (Ali et al., 2004; Jain, Dhawan, Malhotra, & Singh, 2003; Majumder & Mazumdar, 2002; Wakabayashi, Chun, & Huber, 2000). As a result, although it should be taken into account that a higher PME activity quantified in vitro may not necessarily indicate an effective higher activity in vivo, due, for example, to possible restrictions to the access of the enzyme to its substrate, in CA plums the lower PME activity may be related to the lower DM of its pectic polysaccharides, which should be already de-esterified, justifying the increase in PG activity.

The higher PG activity could be related to the higher content of pectic polysaccharides in the water and imidazole extracts of CA than VV fresh plums (Table 2), as well as a higher content of the pectic polysaccharide extracts of CA plums in all stages of processing (Fig. 1a). This is in accordance with the increase of solubilisation and depolymerisation of pectic polysaccharides, which occur during ripening of the fruits and their correlation with the decrease in fruit tissue firmness (Priya Sethu, Prabha, & Tharanathan, 1996; Taylor, Rabe, Jacobs, & Dodd, 1995; Wakabayashi et al., 2000). Depolymerisation of chelator-soluble pectic polysaccharides during ripening has been reported in many fruit types, such as tomato, avocado, kiwi fruit, persimmon, apple, water melon, plum and nashi pear (Redgwell, MacRae, Hallett, Fischer, Perry & Harker, 1997), as well as in olive (Mafra et al., 2001, 2006b). For both PME and PG, the activity was found to be mainly due to ionically and covalently bound cell wall fractions, indicating that these enzymes might be acting primarily on the cell wall. Also, the much higher activity of PG found for CA orchard plums indicates that these plums might be at a more advanced stage of ripening than is indicated by the soluble solids content and titratable acidity measurements.

In order to evaluate the extent of pectic polysaccharides that diffused to the sucrose syrup with the candying process, the amount of polymeric material and its sugar composition was analysed for both VV and CA samples.

3.4. Polysaccharides of sucrose syrup

Table 7 shows that the material recovered after exhaustive dialysis with a large number of water exchanges of the sugar syrup of

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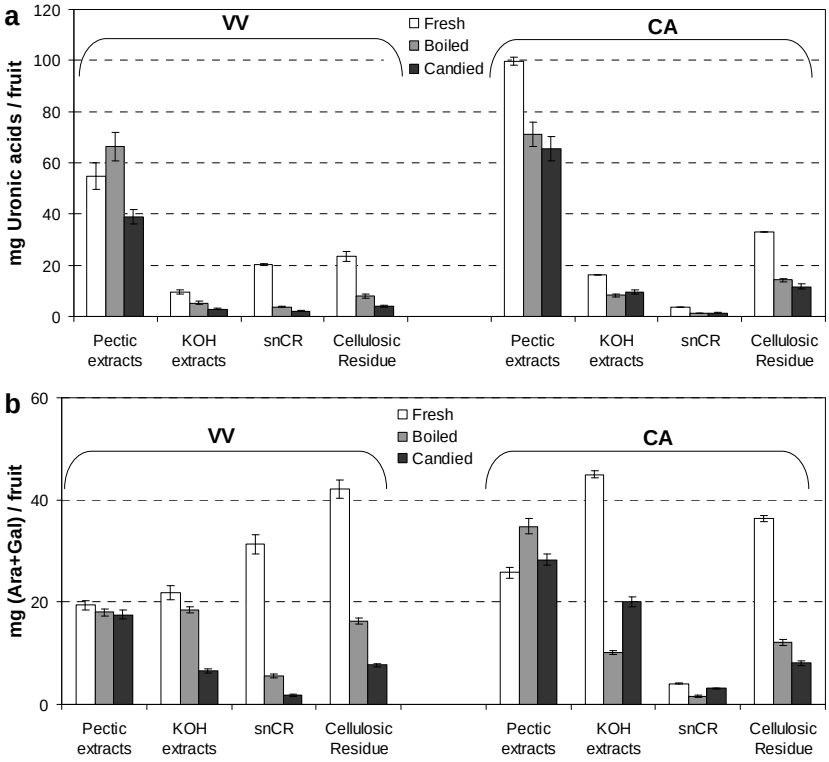

Fig. 1. Uronic acids (a) and Ara + Gal (b) content in combined cell wall extracts and cellulosic residue, expressed as mg per fruit, in fresh, boiled, and candied plums from Vila Viçosa (VV) and Cano (CA) orchards.

Table 5
Degree of methylesterification (DM) and acetylation (DA) of AIR and water and imidazole extracts of fresh, boiled, and candied plums of Vila Viçosa (VV) and Cano (CA) orchards

Extract	Orchard	DM (%)	DA (%)
Fresh			
AIR	**VV**	57 ± 1^{a}	6 ± 1^{a}
	CA	38 ± 2^{b}	5 ± 1^{a}
Water	**VV**	51 ± 5^{a}	5 ± 0^{a}
	CA	36 ± 2^{b}	7 ± 0^{a}
Imidazole	**VV**	65 ± 3^{c}	-
	CA	75 ± 2^{d}	-
Boiled			
AIR	**VV**	54 ± 4^{a}	10 ± 1^{b}
	CA	39 ± 2^{b}	11 ± 0^{b}
Water	**VV**	83 ± 3^{e}	15 ± 1^{c}
	CA	66 ± 2^{c}	15 ± 2^{c}
Imidazole	**VV**	50 ± 5^{a}	-
	CA	40 ± 2^{b}	5 ± 1^{a}
Candied			
AIR	**VV**	44 ± 1^{b}	3 ± 0^{d}
	CA	26 ± 2^{f}	13 ± 0^{c}
Water	**VV**	70 ± 1^{g}	6 ± 1^{a}
	CA	44 ± 2^{b}	12 ± 1^{c}
Imidazole	**VV**	62 ± 1^{c}	9 ± 1^{b}
	CA	39 ± 0^{b}	10 ± 2^{b}

Mean ± standard deviation (n = 3).
Within columns, means with the same superscript letter are not significantly ($p > 0.05$) different.

VV plums (1.3 mg/ml) was lower than the amount recovered in CA (2.1 mg/ml). This material had a carbohydrate content of 48% for VV and 43% for CA, and was composed mainly of UA, Glc, Ara, and Gal. Man and Rha were also present. This sugars composition suggests that, in both samples, pectic polysaccharides account for 60–66% of the total polysaccharides recovered. The average ratio of UA/(Ara + Gal) was 2 for VV and 1 for CA, and the DM was 68% in VV samples and only 38% in CA. These results infer that the pectic polysaccharides present in the sugar syrup of VV plums contained less branched and more methylesterified pectic polysaccharides. The extent of branching and DM were comparable

Table 6
Pectin methylesterase (PME), polygalacturonase (PG), and cellulase (Cel) activities (U) in fresh plums of Vila Viçosa (VV) and Cano (CA) orchards

Enzyme	Vila Viçosa	Cano
PME		
Soluble	-	-
Ionic	0.50 ± 0.04	0.63 ± 0.05
Covalent	0.74 ± 0.01	0.28 ± 0.04
Total	1.24 ± 0.05^{a}	0.90 ± 0.09^{b}
PG		
Soluble	4 ± 1	23 ± 9
Ionic	-	-
Covalent	16 ± 4	101 ± 18
Total	19 ± 5^{c}	124 ± 28^{d}
Cel		
Soluble	-	tr
Ionic	tr	tr
Covalent	0.26 ± 0.01	0.30 ± 0.01
Total	0.26 ± 0.01^{e}	0.31 ± 0.01^{f}

tr, trace amount.
Mean ± standard deviation (n = 3).
Means with the same superscript letter are not significantly ($p > 0.05$) different.

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Table 7
Yield, sugar composition, and degree of methylesterification of sucrose syrup for candied plums from Vila Viçosa (VV) and Cano (CA) orchards

Orchard	Yield[a] (mg/ml)	Sugars (mol%)								Total (mg/g)	DM (%)
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA		
VV	1.3	2 ± 0	–	13 ± 0	2 ± 0	6 ± 0	7 ± 1	27 ± 2	44 ± 2	482	68 ± 5
CA	2.1	3 ± 0	–	20 ± 2	2 ± 0	5 ± 0	7 ± 0	34 ± 1	30 ± 3	432	36 ± 3

Mean ± standard deviation (n = 4).
[a] Yield is expressed in mg of dry weight material per ml of sucrose syrup.

to those observed in the pectic extracts of the fruit, which is in accordance with the hypothesis of diffusion of the cell wall polysaccharides from the fruits to the sucrose syrup during candying.

The presence of highly methylesterified pectic polysaccharides may be responsible for the high viscosity of the sugar syrup, when compared to the viscosity of a solution of sucrose with the same concentration. In fact, the viscosity of the plums' sugar syrup, measured at a shear rate of 280 s^{-1}, was 3.0 Pa, whereas the viscosity of the sucrose solution was 0.010 Pa. These results are in accordance with the results reported by Peiró et al. (2006), which showed a loss of UA from grapefruit tissues during osmotic dehydration, and a progressive increase of the pectin content and viscosity of the solution. Beyond pectic polysaccharides, other fruit compounds were also observed to diffuse. to the syrup, namely, volatile compounds (Nunes, Coimbra, Saraiva, & Rocha, 2008)).

3.5. Hemicellulosic polysaccharides and cellulase activity

In order to evaluate the changes occurring to the hemicellulosic polysaccharides during candying, the partially depectinated AIR was extracted with KOH solutions of increasing strength until a cellulosic residue was obtained. Upon neutralisation of the KOH extracts, the precipitated polymeric material was removed from the supernatant solutions. The sugars composition of the soluble and precipitated material was determined for all KOH extracts of fresh, boiled, and candied plums (Tables 2–4) and identical data

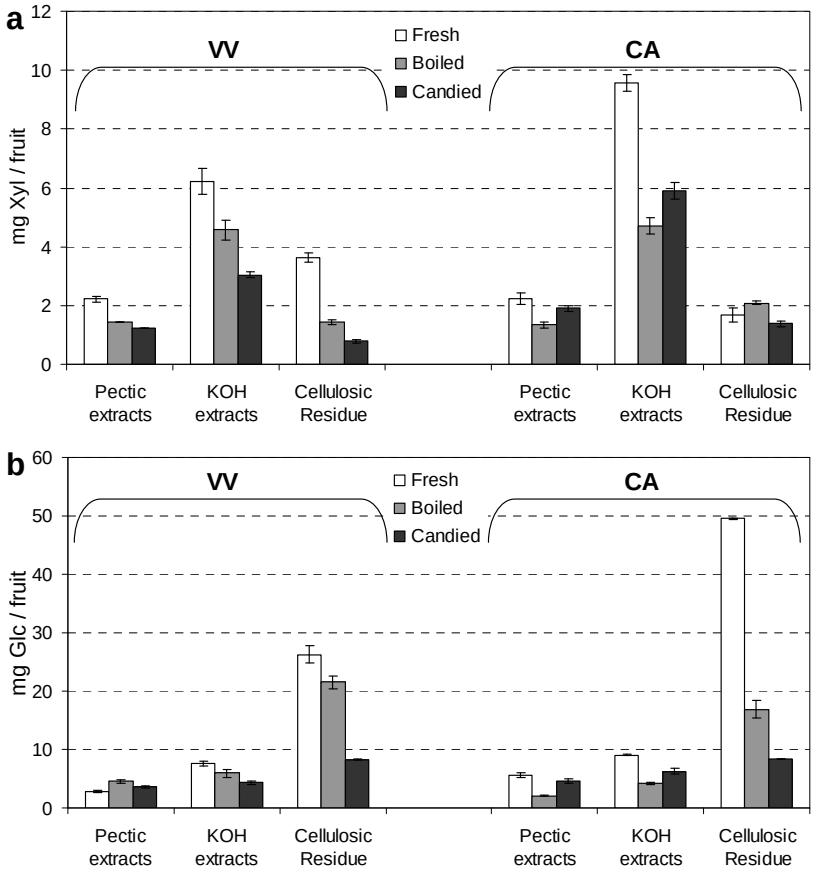

Fig. 2. Xylose (a) and glucose (b) content in combined cell wall extracts and cellulosic residue, expressed as mg per fruit, in fresh, boiled, and candied plums from Vila Viçosa (VV) and Cano (CA) orchards.

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were obtained. The majority of the precipitated material was poor in sugars when compared to the supernatants, containing high amounts of UV-absorbing material, as observed for other fruits (Coimbra et al., 1994). Xyl was the main sugar present in soluble 0.5 M KOH extracts. The presence of a higher amount of Xyl than Glc is diagnostic of xylans (Mafra et al., 2001). However, the presence of Glc, Gal, and traces of Fuc, apart from Xyl, is diagnostic of xyloglucans. These polymers, although in small amount, occur in all KOH fractions. Soluble 1 M, 4 M, and 8 M KOH extracts were mainly composed of Ara and Gal, with lower contents of UA, Glc, and Xyl. The high amounts of Ara and Gal are indicative of the presence of arabinogalactans. The presence of lower quantities of mannans was indicated by the occurrence of Man in the KOH extracts, principally in soluble 4 M KOH. Based on these results, it can be inferred that the hemicelluloses constituent of the cell walls of plums are xylans, xyloglucans, arabinogalactans-rich polymers, and, in a minor quantity, mannans.

On a fruit basis, the amount of Xyl and Glc decreased with boiling (Fig. 2) and this decrease was higher in CA than in VV. After candying, a decrease in the amount of these sugars was also observed in VV plums, while in CA plums the decrease was only found in CR. These results suggest the solubilisation of the polysaccharides with processing. This different behaviour of solubilisation of these sugars is in accordance with the previous observation that the loss of cell wall polysaccharides in VV occurs mainly during the candying process whereas those of CA occur mainly during boiling.

In the cellulosic residue (CR), the main sugar present was Glc, which accounted for 24 and 39 mol% of CR sugars in fresh plums from VV and CA, 43 and 35 mol% in boiled plums, and 38 and 22 mol% in candied plums from VV and CA, respectively (Tables 2–4). The non-cellulosic Glc accounted for 10% of total Glc of the CR of fresh plums from VV, and 5% in CA. An increase of non-cellulosic Glc yield in CR was observed in boiled and candied fruits to 15% in VV and 22% in CA. Based on these results, the amount of cellulose can be estimated to be 24 and 47 mg/fruit for VV and CA fresh plums, respectively, 18 and 13 mg/fruit in boiled plums, and 7 mg/fruit in both processed plums. These results showed that upon boiling, cellulose degradation was observed in the fruits from both orchards, whereas a higher extent of degradation was observed in CA samples.

In order to study the importance of the activity of cellulase (Cel) of fresh plums to the observed degradation of cellulose, cellulase activity was quantified in both VV and CA fresh samples (Table 6). The Cel activity was significantly higher in CA than in VV plums. This higher activity could be related to the higher demethylesterification of pectic polysaccharides by the action of PME, since this enzyme could directly or indirectly assist other enzymes, besides PG, mainly by promoting accessibility of the enzymes, like Cel, to their substrates (Ali et al., 2004). The observed activity of Cel is in accordance with the results obtained for the analysis of cell wall polysaccharides. Cel could degrade both cellulose and the β-1,4-glucan backbone of xyloglucan, which leads to extensive polysaccharide depolymerisation (Iannetta, van den Berg, Wheatley, McNicol, & Davies, 1999; Priya Sethu et al., 1996; Wakabayashi et al., 2000). The higher recovery in CA plums of polysaccharides with KOH solutions seems to be associated to the higher Cel activity of these fruits which promoted solubilisation of the xyloglucans (Fig. 2). This is also in accordance with the higher amount of cellulosic Glc in VV when compared to CA plums.

4. Concluding remarks

Plum cell wall polysaccharides are composed mainly of pectic polysaccharides and cellulose. During the boiling step of the processing to Ameixa d'Elvas these polymers are degraded and solubilised. This may explain the observed decrease of cell wall adhesion and loss of firmness of the tissues. The plums' pectic polysaccharides are highly esterified. Their gelation in the presence of sucrose is responsible for the recovery of the fruit's consistency upon candying. During the candying process diffusion of these methyesterified pectic polysaccharides to the sucrose syrup increase the syrup viscosity, which may explain the retention of several fruit volatile compounds, which contribute to its aroma.

The higher solubilisation of CA plums cell wall polysaccharides after boiling, which was not observed in VV plum, should explain the lower firmness of these plums after candying. This higher susceptibility to the heat treatment could be related to the higher activity of CA plums cell wall enzymes. Although these plums have been produced in the same geographic region and had similar degree of ripening when evaluated by the soluble solids content and titratable acidity, the differences found seem to be related to different stages of ripening not highlighted by these parameters. The analysis of the cell wall polysaccharides and enzymes of CA plums show that these fruits might be in a more advanced stage of ripening than VV. A more adequate assessment of the ripening stage of the fresh fruits, perhaps based on cell wall polysaccharides and/or enzymes seems to be essential to avoid the lower texture quality of ''Ameixa d'Elvas" produced using CA plums.

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CAPÍTULO X

EFFECT OF RIPENING ON MICROSTRUCTURE AND TEXTURE OF "AMEIXA D'ELVAS" CANDIED PLUMS

Effect of ripening on microstructure and texture of "Ameixa d'Elvas" candied plums

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Abstract

The plums used to produce a traditional candied product, "Ameixa d'Elvas", were obtained from two orchards, Vila Viçosa (VV) and Cano (CA). These orchards were selected because the fruits have been behaving differently: 1. The day of harvesting for candying, established by the total soluble solids and titratable acidity, is one week earlier in VV; 2. VV yield candied plums with good texture properties whereas CA give poor processed fruits. In order to understand the origin of these differences, fruits from both orchards were harvested in the day established as the harvesting day for VV (day 1) and for CA (day 8). Comparable texture properties were obtained in firmness, rigidity and deformation work between the VV fresh plums harvested in day 1 and the CA plums harvested in day 8 but lower in CA when the flesh was analysed separately, which agreed with the activity of pectin methylesterase, polygalacturonase and cellulase. The increase of the intercellular area of parenchyma cells and the decrease in cell area caused by boiling, which resulted in a pronounced loss of texture properties, were partially recovered after the immersion of the fruits in the sucrose syrup. The plums from CA harvested in day 8 had a more pronounced degradation with boiling and lower recovery of cells shape, size and texture characteristics than those of VV harvested in day 1. Upon candying, similar properties were observed for the fruits harvested in the same day: good candied products were observed for VV and CA fruits harvested in day 1 and poor candied products were observed for VV and CA fruits harvested in day 8. This work shows that the characteristics of the flesh of the fresh fruits are a key parameter to define the texture properties of the candied plum. The establishment of the harvesting moment for candying should take into account the changes that occur in the flesh of the plums during ripening.

Keywords: *Prunus domestica*; Green Gage; Microstructure; Histocytological studies; Pectin methyl esterase; Polygalacturonase; Cellulase

1. Introduction

"Ameixa d'Elvas" is a Protected Designation of Origin (PDO) recognized by the European Union for a candied plum (*Prunus domestica* L.) from Alto Alentejo (South-East of Portugal), obtained by a traditional candying process. Only the fruits of 'Green Gage' variety, 'Rainha Cláudia Verde', can be utilized to produce candied "Ameixa d'Elvas". Briefly, the candying process consists in boiling the intact plums in water, followed by immersion in sugar syrup solutions of increasingly concentration up to 75 ºBrix. The ripening stage of the plums, when harvested for candying, seems to be one of the most important factors influencing their final textural properties. For this reason, the plums used to produce "Ameixa d'Elvas" are harvested in a well defined maturation point evaluated by the total soluble solids (16 ºBrix) and titratable acidity (1.0 meq malic acid/100 g fruit flesh weight).

The major textural changes resulting in the softening of fruits are due to enzyme-mediated alterations in the composition and structure of cell wall polysaccharides, such as pectic polysaccharides and cellulose, which leads to their partial solubilisation (Waldron, Smith, Parr, Ng & Parker, 1997). The activity of certain enzymes is related with cell wall polysaccharides changes along the ripening process. Cell walls are complex in composition and structure and, thus, it is unlikely for any particular enzyme alone to be able to significantly modify their properties. A combined action of a number of enzymes acting synergistically is a more plausible cause for the occurrence of the changes needed to impact any significant texture changes on fruits (Ali, Chin & Lazan, 2004). Enzymes such as pectin methyl esterase (PME), polygalacturonase (PG), and cellulase (Cel) are among the enzymes generally recognized as having a crucial role on fruits texture during ripening. The quantification of the activity of these enzymes is important to ascertain their contribution for the changes in texture due to the alteration of cell wall polysaccharides (Wakabayashi, 2000; Ali et al., 2004; Prasanna, Prabha & Tharanathan, 2007).

The main contributors to textural losses during thermal processes are cell degradation, with the concomitant cell wall separation and final collapse of the pectin network, affecting extensively cell adhesion. Cell disruption influences also a number of

biochemical and chemical reactions, allowing substrate/enzyme and reagents contact, that also cause changes of texture (Aguilera, 2005). Cell structure modification of fruits caused by heat treatment has been shown to increase the flow rate of sucrose diffusion during fruits osmotic dehydration (Nieto, Salvatori, Castro & Alzamora, 1998), which can also be relevant during the candying process.

In this work, plums from two orchards, Vila Viçosa (VV) and Cano (CA), within the PDO region, were studied. These two different orchards were selected because of the different behaviour of the plums during ripening and candying process. CA plums had usually one week delay in ripening stage, evaluated by total soluble solids and titratable acidity, in relation to VV plums. Moreover, it is known that plums from CA orchard, although presenting the adequate maturity stage, result in a candied product of low or even no commercial use due to appreciable loss of tissue consistency and skin disruption (Nunes, Santos, Pinto, Lopes-da-Silva, Saraiva & Coimbra, 2008a). Contrarily to CA plums, the fruits from VV originate a candied product with good textural properties, when harvested at the appropriate maturity stage. In these fruits, differences in the structure of their cell wall polysaccharides and related enzyme activities were noticed (Nunes, Saraiva & Coimbra, 2008b). In order to understand the origin of these differences, fruits from both orchards were harvested in the day established as the harvesting day for each orchard. The histocytological and texture properties of fresh plums were evaluated and the activity of the enzymes having catalytic action on cell wall (PME, PG, and Cel) was quantified to ascertain their influences in the texture of the plums. The plums from both orchards were candied and the texture properties of the tissues were measured in order to evaluate objectively the influence of the stage of ripening of the fruit at harvesting on the final texture quality of the candied products.

2. Materials and methods

2.1. Plant material and sample preparation

Plums (*Prunus domestica* L.) of ‘Green Gage’ variety, ‘Rainha Cláudia Verde’, were supplied by Fruteco-Fruticultura Integrada, Lda. (Borba, Portugal). They were collected in 2005 in two orchards, Vila Viçosa (VV) and Cano (CA), within the PDO region.

a) VV and CA plums were collected in the day established as the harvesting day for the candying process, when the fruits reach 16 ºBrix and a titratable acidity of 1.0 meq malic acid/100 g of fresh weight. The fruits harvested in day 1 (19th July), the harvesting day for VV plums, were designated VVf1 and CAf1, and the fruits harvested in day 8 (26th July), the harvesting day for CA plums, were designated VVf8 and CAf8, where f stands for fresh, and 1 or 8 stand for the collection day. Plums were brought immediately to the laboratory, being the histocytological and texture analyses carried out on the same day.

b) VV plums were also collected 6 days before and 4 days after the first harvesting, on 13th and 22nd July, respectively.

c) Fresh plums harvested on day 1 and 8 from both orchards were candied according to the traditional process, at the day of harvesting, on a laboratory scale. First, the plums were boiled in water for about 15 min (water:fruit ratio of 4:1), until their floatation occurred (some boiled plums were withdrawn to study the effect of the boiling step on texture and cell properties). After, they were immersed in a 60 ºBrix sucrose syrup (syrup:fruit ratio of 2:1). In the following day, the sugar solution was concentrated by addition of sucrose powder and heating to reach 65 ºBrix and to 75 ºBrix after 7 days. The plums were kept for two months in the 75 ºBrix sugar syrup, at room temperature, being the concentration of the sugar syrup adjusted by heating when necessary (2 or 3 times), due to its hygroscopicity.

2.2. Total soluble solids, pH, and titratable acidity

Total soluble solids, pH, and titratable acidity were determined in fresh plums. Plum juice was obtained by squeezing plums flesh and filtration through a glass fibre filter (Whatman GF/C). Total soluble solids and titratable acidity were determined on the filtrate. Total soluble solids (ºBrix) were determined by measuring the refractive index of the juice with a hand refractometer (ATC-1E, Atago Co. Lda., Japan). Titratable acidity was measured using 6 g of juice diluted with 50 mL of distilled water, by titration with 0.1 M NaOH to an endpoint of pH 8.1, using an automatic pH–stat (Crison micro TT2022, Alella, Spain) and was expressed as meq of malic acid/100 mL juice.

2.3. Histocytological analyses

The histocytological analyses were performed on plums from VV and CA orchards without any processing (VVf1, VVf8, CAf1, and CAf8), boiled (VVb1, VVb8, Cab1, and Cab8), and candied (VVc1, VVc8, CAc1, and CAc8) plums (b and c stand for boiled and candied, respectively).

Material preparation and fixation, for light and scanning electron microscopy (SEM) analysis, was performed as previously described by Pinto (2007). Briefly, samples were fixed in 2.0 mL/100 mL glutaraldehyde in 0.04 mol/L of PIPES buffer pH 7.6 (Duchefa, Haarlem, The Netherlands) overnight at 4ºC and then washed in PIPES. Tissues were transferred to 1.0% (w/v) osmium tetroxide in PIPES buffer and dehydrated using increasingly concentrated ethanol solutions (30 - 100%, v/v).

For light microscopy, samples were embedded in an epoxi resin (Embed-812). Semi-thin sections (1 μm) were stained with aqueous 1% (w/v) methylene blue. Samples were analysed with a Nikon Eclipse 80i light microscope (Nikon Co, Kanagawa, Japan) and photographs were taken using a Leica DC 200 digital camera (Leica Microsystems AG, Germany).

For SEM analysis, ethanol dehydrated samples were further dehydrated by successive immersions in acetone solutions of increasingly concentration (30 - 100%, v/v) and finally in a critical point device (Baltec CPD 030, USA) using CO_2 as transition agent. Samples were fixed on steel supports and coated with gold using a JEOL metalizer (FFC-1100, Japan) at 1100-1200 V, 5 mA for 10 min. Samples were observed in a scanning electronic microscope (Hitachi, S4100, Japan) at 20 kV.

Microstructure images were analysed using the image analysing program UTHSCSA Image Tool, version 3.00 (University of Texas Health Science Center, USA). Cell axis, area, and intercellular area were estimated based on optical microscopic and SEM images, at least, on three images from different pieces of each sample and five measurements were made in each image, resulting in a total of 15 measurements, for each of the three quantified parameters.

2.4. Fruit texture analyses

Fresh (VVf1, VVf8, CAf1, and CAf8), boiled (VVb1, VVb8, CAb1, and CAb8), and candied (VVc1, VVc8, CAc1, and CAc8) plums were used for texture characterisation. Puncture tests were performed using a TA-Hdi Texture Analyser (Stable Micro Systems, Godalming, UK), equipped with a 5 kg load cell and a 2 mm diameter cylindrical stainless steel probe. Penetration was done at 1 mm/s to a depth of 15 mm. Each plum was cut by the longitudinal line and one half was analysed with skin and another half without skin, with puncturing carried out from the external plums surface, in both cases. At least six different fruits of each sample were used, and each half was punctured at five different locations, one on the middle and four around the middle, on the corners of a square, 7-10 mm apart from the middle punctured point. Firmness (the peak maximum force), rigidity (the maximum initial slope of the force-displacement curve obtained during the puncture tests), and the deformation work applied to the sample (calculated as the area beyond the force-displacement curve until the maximum force), during the downstroke puncture, were used to characterise the textural properties of the samples.

2.5. Preparation of cell wall material

Fresh plums (500 g) from both orchards (VVf1, VVf8, CAf1, and CAf8) were dispersed in ethanol (2 L) at a final concentration of 85% (v/v) and boiled for 10 min. The mixture was cooled, filtered through a glass fibre filter (Whatman GF/C) and the residue was then washed with diethyl ether and allowed to dry at room temperature. The dried material constitutes the alcohol insoluble residue (AIR).

2.6. Carbohydrate analysis

Neutral sugars were obtained by sulfuric acid hydrolysis (Selvendran, March & Ring, 1979) and analysed after conversion to their alditol acetates by GC, using 2-deoxyglucose as internal standard (Coimbra, Delgadillo, Waldron & Selvendran, 1996). A Carlo Erba 6000 GC apparatus with split injector and a FID detector was used, equipped with a 30 m

column DB-225 (J&W) with i.d. and film thickness of 0.25 mm and 0.15 □m, respectively. The oven temperature program used was: initial temperature 200 ºC, a rise in temperature at a rate of 40 ºC/min until 220 ºC and then 220 ºC for 14 min, followed by an increase until 230 ºC at rate of 20 ºC/min, being this temperature maintained for 1 min. The injector and detector temperatures were, respectively, 220 and 230 ºC. The flow rate of the carrier gas (H_2) was set at 1 mL/min.

Uronic acids (UA) were quantified by a modification (Coimbra et al., 1996) of the 3-phenylphenol colorimetric method (Blumenkrantz & Asboe-Hansen, 1973), using a calibration curve made with D-galacturonic acid. Samples were prepared by hydrolysis in 0.2 mL of 72% H_2SO_4 for 3 h at room temperature followed by 1 h in 1 M H_2SO_4 at 100 ºC. The hydrolysis of all samples was done in duplicate and each sample was injected twice.

2.7. Quantification of enzymatic activity

The enzymatic activity of the enzymes PME, PG, and Cel was quantified in all fresh plums harvested from both orchards. The enzymatic extract was obtained based on a procedure described by Denès et al. (2000). One hundred grams of plums pulp was homogenized in 100 mL 0.2 M Tris(hydroxymethyl)-aminomethane buffer (Tris buffer), at pH 7.0, containing 500 mg/L sodium metabisulfite ($Na_2S_2O_5$) and 1% polyvinylpolypyrrolidone (PVPP). After extraction (2 h at 4 ºC), the suspension was centrifuged at 20,000*g* for 15 min at 4 ºC and the supernatant obtained was used as the source of soluble fraction (SF) enzymatic extract. The pellet was dispersed on the same buffer, but containing 1 M NaCl, and stirred for 2 h at 4 ºC, followed by centrifugation (20,000*g* for 15 min). The supernatant obtained was used as the source of cell wall ionically-linked fraction (IF) enzymatic extract. The remaining pellet was used to determine the activity of the cell wall strongly-linked enzymatic fraction, usually called covalently-linked enzymatic fraction (CF). Enzymatic activities were quantified for SF, IF, and CF, because the relative activity of the three forms and the different interaction of the three forms with the cell wall components, can imply a different mobility capacity and a different capacity to act on cell wall polysaccharides. Enzymatic activity was determined

in triplicate and expressed on plum pulp weight basis, for all enzymes. Total activity was calculated by summing up the average activity of the three fractions.

Pectin methyl esterase (PME) activity was measured by the continuous recording of the titration of carboxyl groups formed on a pectin solution using an automatic pH–stat (Crison micro TT2022, Alella, Spain) and a 0.01 N NaOH solution (Nunes, Castro, Saraiva, Coimbra, Hendrickx & Van Loey, 2006). Assays were performed with a 3.5 mg/mL apple pectin solution (DE 75%, 30 mL) containing 0.117 M NaCl at pH 7.0 and 25 ºC. Activity was quantified by adding to 15 mL of pectin solution, 0.5 mL of enzyme extract, for SF and IF extracts, or approximately 1 g of remaining pellet for the CF. One unit (U) of PME activity was defined as the amount of enzyme necessary to generate 1 µmol of carboxyl groups per min, under the previous mentioned assay conditions.

Polygalacturonase (PG) activity was quantified according to the method described by Gross et al. (1982), which is based on the measurement of reducing groups formed using polygalacturonic acid as substrate. The substrate solution contained 0.4% (w/v) polygalacturonic acid in 0.05 M sodium acetate buffer (pH 4.5) and the reaction was carried out by adding 0.2 mL of enzyme extract (SF and IF) or 0.1 g (CF), followed by incubation at 35 ºC for 10 min. The reaction was stopped with 2 mL of 10 mM borate buffer at pH 9 and 0.4 mL of 1% (w/v) 2-cianoacetamide. The mixture was put in a boiled water bath during 10 min and after cooling the absorbance at 276 nm was measured. The amount of reducing groups formed was determined using a calibration curve made with D-galacturonic acid and the enzyme activity was expressed as nmol of galacturonic acid released per min. One unit (U) of PG activity was defined as the amount of enzyme that originated 1 nmol of reducing groups per min.

The activity of cellulase (Cel, EC 3.2.1.4) was measured by determining the viscosity of a carboxymethylcellulose substrate solution, before and after the action of the enzyme. Viscosity was measured using a Cannon-Fenske capillary viscometer (75 mm), and the substrate solution consisted of a 0.1 % (w/v) carboxymethylcellulose in 0.1 M acetate buffer at pH 4 (Lohani, Trivedi & Nath, 2004). In a standard assay 0.50 mL of acetate buffer, 0.50 mL of enzymatic extract, adequately diluted (SF and IF), or a proper amount (typically about 1 g) of solid residue and more 0.50 mL of buffer (CF), were added to 4 mL of substrate solution. The enzymatic reaction took place at 30 ºC during 60 min. Activity of

Cel was expressed as the relative decrease of viscosity, in relation to an assay without enzyme. One unit (U) of Cel activity was defined as the amount of enzyme that caused a 1% reduction of viscosity per min.

2.8. Statistical analysis

Quantitative analyses are presented as mean values and the reproducibility of the results is expressed as standard deviation or coefficient of variation in tables and as standard error bars in figures. Statistical analysis of the experimental results was carried out based on *F*-test and Student's *t* test (Microsoft Excel, Microsoft Corporation, Redmond, USA). Significant differences were considered at the level of $p<0.05$.

3. Results and discussion

According to Table 1, VV plums reached 16 ºBrix and a titratable acidity of 1.0 meq of malic acid /100 g of fresh weight, the maturity parameters considered optimum to harvest these fruits for candying purposes, in 19th July (day 1) whereas CA achieved these values only at 26th July (day 8). On 19th July, the plums from CA orchard showed only 13.7 ºBrix and a titratable acidity of 1.18, which shows an earlier maturity stage than that presented by VV fruits on 13th July, almost one week before its optimum day for harvesting. This difference, which has been already noticed in previous years (Nunes et al., 2008a,b), shows that VV plums might be at a more advanced stage of ripening than CA.

Table 1 – Weight, total soluble solids (ºBrix), titratable acidity (meq of malic acid/100g), and pH of fresh plums from Vila Viçosa (VV) and Cano (CA) orchards.

Sample	*Day*	*Weight (g)*	*Total Soluble Solids (ºBrix)*	*Titratable Acidity (meq of malic acid/100 g)*	*pH*
Vila Viçosa					
VVf-5	13 Jul 05	35 ± 1^{a}	14.5 ± 0.1^{a}	1.14 ± 0.03^{a}	3.19 ± 0.01^{a}
VVf1	19 Jul 05	35 ± 1^{a}	16.1 ± 0.1^{b}	1.03 ± 0.01^{b}	3.22 ± 0.01^{a}
VVf4	22 Jul 05	36 ± 1^{a}	17.1 ± 0.2^{c}	1.00 ± 0.04^{b}	3.22 ± 0.01^{a}
VVf8	26 Jul 05	36 ± 1^{a}	19.6 ± 0.1^{d}	0.90 ± 0.01^{c}	3.32 ± 0.02^{b}
Cano					
CAf1	19 Jul 05	33 ± 1^{b}	13.7 ± 0.1^{e}	1.18 ± 0.01^{a}	3.16 ± 0.01^{a}
CAf8	26 Jul 05	33 ± 1^{b}	15.7 ± 0.2^{b}	1.04 ± 0.01^{b}	3.27 ± 0.02^{b}

Mean ± standard deviation (n=3). Within columns, means with different superscript letters are significantly ($p<0.05$) different.

3.1. Fresh plums analysis

3.1.1. Histocytological and texture analysis

In both orchards, VV and CA, parenchymatous tissue of fresh plums harvested at the day 1 (VVf1 and CAf1) showed isodiametric parenchyma cells with large dimensions (diameter of 2.7-2.9 μm and area of 5.9-6.1 μm^2) and a well defined middle lamella with an intercellular area of 0.02 μm^2 (Table 2). Parenchyma cells with a more irregular shape and with a loss of cell-to-cell contact were observed in day 8, representing a decrease in cell area of 33-35% and an increase in intercellular area of 66% for VV and 34% for CA (Table 2) with evident cell separation (Figure 1). With the evolution of the ripening stage, fruits show progressively more cells with irregular shapes and larger intercellular areas (Redgwell, MacRae, Hallett, Fischer, Perry & Harker, 1997). Vascular strands, composed of xylem and phloem tissues, appeared diffusely distributed in the parenchyma and no changes were observed with ripening or between orchards. Comparing the VVf1 fruits with CAf8, significant histocytological differences were only observed in cell area (25% higher for VVf1). During fruits ripening, there is a change in the mode of tissue failure, from only cell rupture to some cell separation, a process already observed for other fruits (Heyes & Sealey, 1996; Mafra et al., 2001).

Table 2 – Cell diameter, area, and intercellular area of parenchymatous cells of fresh (VVf and CAf), boiled (VVb and CAb) and candied (VVc and CAc) plums from Vila Viçosa and Cano harvested at day 1 and day 8.

Sample	*Cell diameter (μm)*	*Cell area (μm^2)*	*Intercellular area (μm^2)*
Fresh			
VVf1	2.93 ± 0.47^{a}	6.08 ± 0.68^{a}	0.02 ± 0.00^{a}
VVf8	2.11 ± 0.45^{a}	3.94 ± 0.62^{b}	0.05 ± 0.01^{b}
CAf1	2.67 ± 0.63^{a}	5.88 ± 0.73^{a}	0.02 ± 0.00^{a}
CAf8	2.49 ± 0.27^{a}	4.59 ± 0.23^{b}	0.03 ± 0.01^{a}
Boiled			
VVb1	2.46 ± 0.24^{a}	4.79 ± 0.18^{b}	0.11 ± 0.03^{c}
VVb8	2.13 ± 0.28^{a}	3.68 ± 0.25^{b}	0.09 ± 0.02^{c}
CAb1	1.97 ± 0.34^{a}	3.51 ± 0.37^{b}	0.21 ± 0.05^{d}
CAb8	1.48 ± 0.17^{b}	2.93 ± 0.09^{c}	0.35 ± 0.05^{e}
Candied			
VVc1	3.80 ± 0.66^{c}	8.66 ± 0.37^{d}	0.04 ± 0.01^{b}
VVc8	3.22 ± 0.34^{c}	7.23 ± 0.37^{e}	0.06 ± 0.01^{b}
CAc1	2.85 ± 0.49^{a}	6.56 ± 0.74^{e}	0.06 ± 0.02^{b}
CAc8	2.27 ± 0.40^{a}	5.15 ± 0.50^{a}	0.05 ± 0.01^{b}

Mean ± standard deviation (n=15). Within columns, means with different superscript letters are significantly (p<0.05) different.

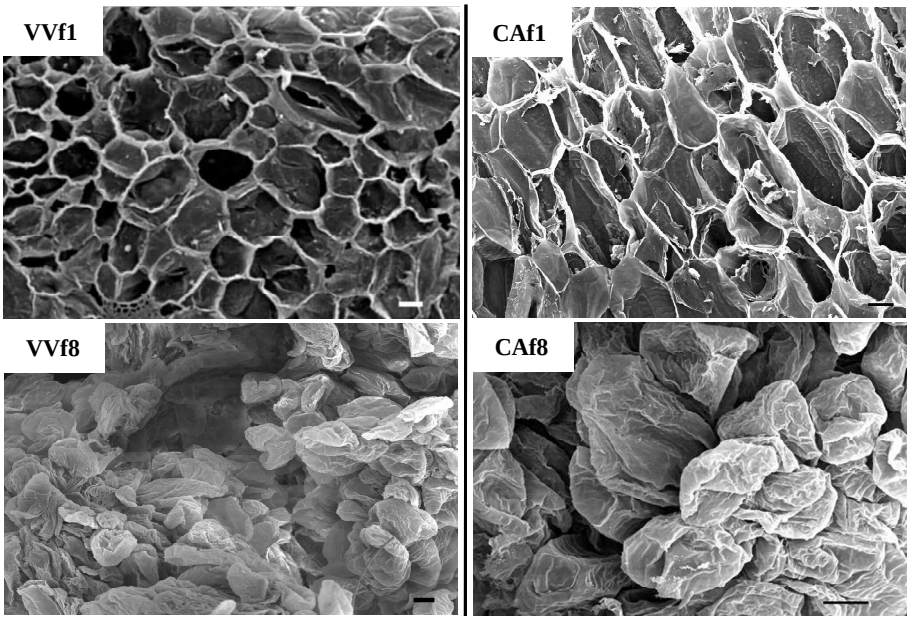

Figure 1. Scanning micrographs of plums from Vila Viçosa and Cano orchard showing regions of parenchymatous cells (bar 100 µm). Plums harvested at day 1 (VVf1 and CAf1) and harvested at day 8 (VVf8 and CAf8).

The values of firmness, rigidity, and deformation work of fruits are associated, respectively, with the cell wall strength, cell wall-to-cell wall adhesion, and cell turgor (Heyes et al., 1996). CAf1 tissues had firmness and deformation work values higher than VVf1 only when the fruits were analysed with skin, but both fruits showed similar flesh texture (Table 3). Also no significant differences were observed between CAf8 and VVf8. Ripening caused a 36-44% decrease in firmness and deformation work for the plums without skin, whereas with skin the decrease was 32% for CA and no significant difference was observed for VV. A significant decrease on rigidity with ripening was only observed in the fruits without skin. Although no significant differences were observed for firmness, rigidity, and deformation work between VVf1 and CAf8 plums analysed with skin, the texture parameters measured only in the flesh revealed that the tissues of VVf1 plums had significantly higher values than CAf8.

Table 3. Firmness, rigidity, and deformation work values of fresh (VVf and CAf), boiled (VVb and CAb) and candied (VVc and CAc) plums from Vila Viçosa and Cano harvested at day 1 and day 8.

Sample	*Firmness (N)*		*Rigidity (N/mm)*		*Work (J x 10^3)*	
	flesh + skin	*flesh*	*flesh + skin*	*flesh*	*flesh + skin*	*flesh*
Fresh						
VVf1	5.01 ± 0.56^{a}	3.66 ± 0.48^{a}	2.28 ± 0.38^{a}	1.66 ± 0.29^{a}	5.07 ± 0.51^{a}	3.66 ± 0.42^{a}
VVf8	4.40 ± 0.47^{a}	2.44 ± 0.29^{b}	1.83 ± 0.37^{a}	1.03 ± 0.27^{b}	4.46 ± 0.46^{a}	2.36 ± 0.28^{b}
CAf1	6.32 ± 0.72^{b}	4.33 ± 0.52^{a}	3.00 ± 0.46^{a}	2.31 ± 0.43^{a}	6.31 ± 0.79^{b}	4.33 ± 0.58^{a}
CAf8	4.30 ± 0.58^{a}	2.36 ± 0.28^{b}	2.43 ± 0.29^{a}	1.36 ± 0.25^{b}	4.34 ± 0.58^{a}	2.44 ± 0.29^{b}
Boiled						
VVb1	0.93 ± 0.18^{c}	0.22 ± 0.05^{c}	0.17 ± 0.03^{b}	0.06 ± 0.01^{c}	1.39 ± 0.27^{c}	0.34 ± 0.08^{c}
VVb8	0.32 ± 0.07^{d}	0.19 ± 0.05^{c}	0.05 ± 0.01^{c}	0.04 ± 0.01^{c}	0.49 ± 0.11^{d}	0.29 ± 0.07^{c}
CAb1	0.91 ± 0.33^{c}	0.47 ± 0.15^{d}	0.14 ± 0.04^{b}	0.09 ± 0.04^{c}	1.36 ± 0.49^{c}	0.71 ± 0.22^{d}
CAb8	0.46 ± 0.09^{d}	0.31 ± 0.12^{d}	0.06 ± 0.01^{c}	0.04 ± 0.02^{c}	0.69 ± 0.14^{d}	0.47 ± 0.18^{c}
Candied						
VVc1	3.46 ± 0.56^{a}	1.60 ± 0.32^{e}	0.42 ± 0.09^{d}	0.26 ± 0.04^{d}	6.91 ± 0.81^{b}	3.20 ± 0.63^{a}
VVc8	2.46 ± 0.35^{e}	0.67 ± 0.16^{d}	0.20 ± 0.05^{b}	0.09 ± 0.03^{c}	4.93 ± 0.71^{a}	1.33 ± 0.33^{b}
CAc1	3.15 ± 0.46^{a}	1.05 ± 0.45^{e}	0.44 ± 0.09^{d}	0.34 ± 0.07^{d}	6.30 ± 0.91^{b}	2.10 ± 0.91^{b}
CAc8	2.35 ± 0.40^{e}	0.93 ± 0.31^{d}	0.19 ± 0.05^{b}	0.21 ± 0.04^{d}	4.69 ± 0.80^{a}	1.86 ± 0.61^{b}

Mean ± standard deviation (n=30). Within columns, means with different superscript letters are significantly (p<0.05) different.

3.1.2. Cell wall enzymatic activity

The activities of PME, PG, and Cel were quantified in VVf1, VVf8, CAf1, and CAf8 plums. In addition, to have a more precise tendency of the activity of these enzymes, assays were also done for the VV plums harvested in an early stage of maturation (VVf-5) and between day 1 and day 8 (VVf4). All enzyme activities were quantified on the soluble (SF), ionically bound (IF), and covalently bound (CF) to cell wall fractions.

PME activity was found mostly in the IF (about two thirds of the total activity) and the evolution of the total activity followed the evolution pattern of the IF (Figure 2). PME activity increased slightly until the 1st day of harvesting for VV plums and then decreased at the 4th and 8th harvesting days to about half of its maximum activity value. In CA orchard, total PME activity also decreased from CAf1 to CAf8. PG activity was mainly observed on the CF and an increase of the activity was observed throughout ripening. Total PG activity increased around 40% from the 1st day to the 8th day of harvesting in both orchards. The increase of PG activity with ripening occurred when the activity of PME began to decrease. This is in agreement with many other works that observed a similar pattern for the evolution of the activity of the two enzymes with ripening in other fruits (Barrett & Gonzalez, 1994; Sethu, Prabha & Tharanathan, 1996; Zhou, Ben-Arie & Lurie, 2000; Majumder & Mazumdar, 2002; Wakabayashi, Hoson & Huber, 2003; Lohani et al.,

2004). PME and PG action on the methyl deesterification and glycoside cleavage of the pectic polysaccharides of the middle lamella might contribute to the increase of the intercellular area observed in the histocytological studies and to the decrease of the measured texture parameters.

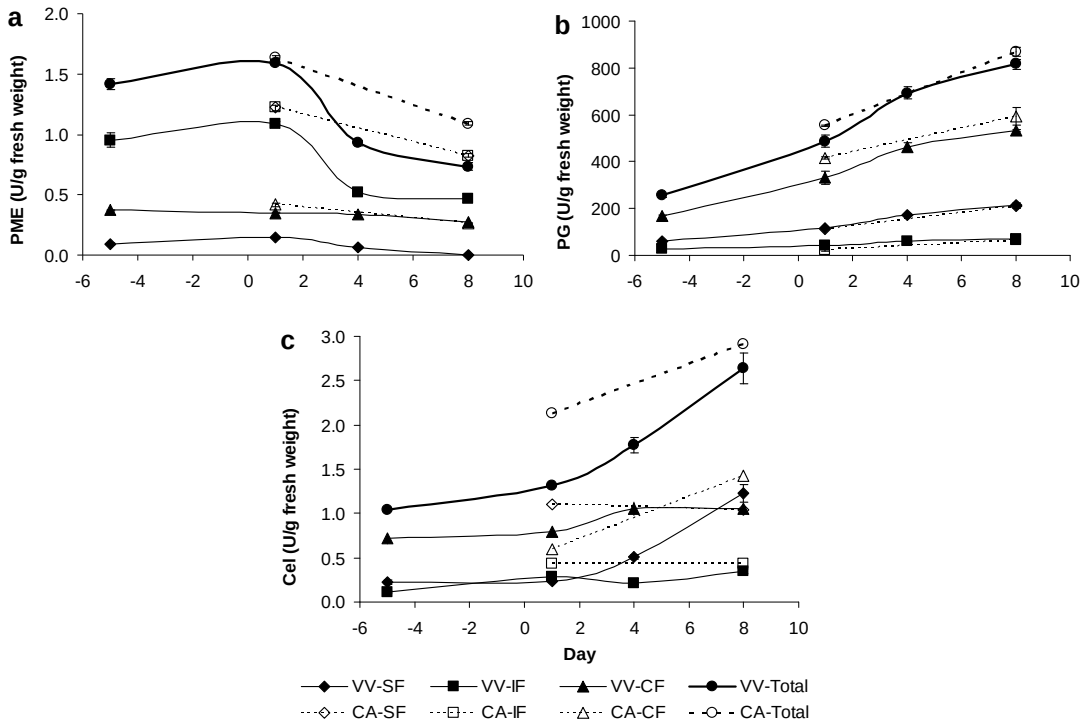

Figure 2. Changes on activity of: a) PME, b) PG, and c) Cel in fresh plums from Vila Viçosa and Cano orchards during ripening. SF, soluble fraction; IF, ionically-linked fraction; CF covalently-linked enzymatic fraction.

The total activity of Cel increased in both VV and CA plums along ripening, occurring mainly in SF and CF. The increase of Cel activity, in addition with the increase of PG activity, is a characteristic of ripened fruits (Fischer & Bennett, 1991; Sethu et al., 1996; Iannetta, van den Berg, Wheatley, McNicol & Davies, 1999; Jain, Dhawan, Malhotra & Singh, 2003; Lohani et al., 2004; Prasanna et al., 2007). Texture loss during ripening has been also correlated with the increase of Cel activity for other fruits (Barrett et al., 1994; Sethu et al., 1996; Lohani et al., 2004; Prasanna et al., 2007).

Plums from CA orchard presented comparable values for total PME and PG activities when compared with those from VV for the same day (Figure 2). The total Cel activity at

the 1st harvesting day was 60% higher for CA plums but similar in day 8. These results show that CA and VV plums were at the same stage of ripening in the same day in what concerns cell wall enzyme activity, despite the differences in the total soluble solids content and titratable acidity previously discussed. Comparing the enzymatic activity of VVf1 with CAf8, the harvesting days to processing the plums according to these parameters, a 32% higher value was obtained for PME activity whereas a 44 and 55% lower values were found for PG and Cel activities, respectively. A similar tendency was previously observed for these plums harvested in 2003 (Nunes et al., 2008b). These results are in accordance with the observed lower texture parameters found for the flesh of CAf8 when compared to VVf1. The increase of PG and Cel activity along ripening should contribute for the loss of texture through the action on cell wall pectic polysaccharides and cellulose, changing their structure and solubilising the cell wall components (Waldron et al., 1997; Ali et al., 2004).

3.1.3. Cell wall composition

The alcohol insoluble residue (AIR) was used to obtain and characterise the polymeric material of fresh plums of both orchards at days 1 and 8 (Table 4). An average of 4-5% of polymeric material was extracted for the four samples of fresh plums analysed. The total sugars content ranged from 20 to 28 mg/g of fruit flesh and increased with ripening about 30% for VV and 20% for CA orchard (Table 4). Uronic acids are the most abundant sugars of the plums cell wall polysaccharides, followed by glucose, arabinose, and galactose. This sugar composition allows to infer that pectic polysaccharides, composed of galacturonic acid, Ara, and Gal, together with cellulose, are abundant in plums. The main effect caused by ripening, in both orchards, is an increase in the AIR of the amount of all sugars, mainly UA (35-40%). The ratio UA/(Ara+Gal), which relates the proportion of galacturonic acid residues of pectic polysaccharides with the proportion of sugars components of their side chains, increased in both orchards, from 1.7 in VVf1 to 2.0 in VVf8, and from 1.5 in CAf1 to 1.8 in CAf8, indicating that the pectic polysaccharides become less branched with ripening. These results, associated with the results of the activity of PME and PG allow to infer solubilisation and depolymerisation of the cell wall pectic polysaccharides with ripening. These changes in cell wall composition are related to

the decrease in fruit tissue firmness during the ripening process observed by the histocytological and texture analyses.

Table 4. Sugar composition of AIR extracts of fresh plums from Vila Viçosa (VVf) and Cano (CAf) orchards, at the 1st and 8th harvesting days.

Sample	*Yield* [a] *(mg/g)*	*Cell wall sugars (mg/g fruit)*								
		Rha	*Fuc*	*Ara*	*Xyl*	*Man*	*Gal*	*Glc*	*UA*	*Total*
Vila Viçosa										
VVf1	43	0.4 ± 0.1	0.3 ± 0.0	2.4 ± 0.1	0.7 ± 0.0	0.5 ± 0.0	2.2 ± 0.1	5.3 ± 0.4	7.8 ± 0.8	19.6
VVf8	48	0.4 ± 0.0	0.2 ± 0.0	3.5 ± 0.2	0.8 ± 0.0	0.6 ± 0.1	3.4 ± 0.2	6.0 ± 0.4	13.5 ± 1.3	28.4
Cano										
CAf1	40	0.4 ± 0.0	0.3 ± 0.0	2.6 ± 0.4	0.9 ± 0.0	0.6 ± 0.1	2.4 ± 0.2	6.7 ± 0.3	7.3 ± 0.6	21.0
CAf8	42	0.4 ± 0.0	0.2 ± 0.0	2.9 ± 0.3	0.8 ± 0.1	0.7 ± 0.0	3.1 ± 0.2	7.2 ± 0.2	11.0 ± 1.3	26.4

[a] Yield is expressed in mg of dry weight material per g of fresh weight plum.
Mean ± standard deviation (n=4).

Between orchards, no significant differences were found for all sugars analysed when samples VVf1 and CAf1 and VVf8 and CAf8 are compared. Although no significant differences have been observed between the AIR of VVf1 and CAf8 in the ratio UA/(Ara+Gal), total sugars were 26% higher in CAf8, as well as the proportion of UA that was 29% higher, suggesting that cell wall polysaccharides of CA plums were in a more advanced stage of ripening than that indicated by the total soluble solids and titratable acidity of the fruit. A similar tendency was previously observed for these plums harvested in 2003 (Nunes et al., 2008b).

3.2. Boiled plums analysis

In order to evaluate the effect of ripening of the fruits in the texture properties of the candied products, fruits of VV and CA orchards at the two harvesting days, 1 and 8, were processed, originating the samples VVb1 and CAb1, and VVb8 and CAb8, respectively. Boiled plums from both orchards showed degradation of parenchyma tissue and vascular strands as shown by Nunes et al. (2008a). The shape of the parenchyma cells (constituted now by cell walls and protoplast debris) became irregular and cell walls appeared detached from neighbouring cells when compared to fresh plums. A decrease in parenchyma cells area with the boiling treatment was observed for all plums analysed, being this reduction

lower for VV (7-21%) than for CA (36-40%) plums at both stages of ripening (Table 2). Boiling increased also intercellular areas significantly for both orchards (45-85% in VV and 86-91% in CA), at the two stages of ripening.

Texture analysis of boiled plums tissues showed a sharp decrease in firmness (82-94%), rigidity (93-98%), and deformation work (72-91%), when compared to the fresh plums for the two orchards and the two days of harvesting (Table 3), in accordance with the observations made for the plums harvested in 2003 (Nunes et al., 2008a). This result was expected, since thermal processing at high temperatures (100 ºC and 15 min in the present study) causes considerable softening of vegetable tissues. This was due to the loss of turgor pressure and cell adhesion of parenchyma cells, as was observed by the decrease of cell area and the increase of the intercellular area by the microstructure analysis (Table 2).

For both orchards, the values obtained for the three texture parameters were significantly higher in the 1st harvesting day (with skin) when compared to the 8th harvesting day. For plums analysed without skin no significant differences were observed with ripening. These results indicate that the extent of texture loss of the plums tissue caused by boiling is affected by the stage of ripening of the fruits, and that the skin has an important influence on the results obtained for the texture parameters. The lower firmness and rigidity obtained for the boiled plums at day 8 are in accordance with the smaller cell area and bigger intercellular area of the plums from the 8th day of harvesting.

Boiled CA plums, comparing to VV, showed a 48-74% higher intercellular area and 20-30% lower cell diameter and 20-27% lower area (Table 2). These differences are particularly pronounced for the 8th day of harvesting. Although for the same day of harvesting the values for the three texture parameters obtained with skin showed no significant differences between orchards, the firmness values measured without skin are different for VV and CA boiled samples harvested in the same day. Also, higher values for the deformation work were observed for CAb1 when compared to VVb1. Firmness, rigidity, and deformation work were significantly higher for VVb1 than for CAb8 plums analysed with skin. It seems that the extensive softness of the flesh in all samples prevent to obtain significant texture differences between them.

3.3. Candied plums analysis

The irregular shape of the boiled plums cells changed to a more spherical shape in candied plums (Nunes et al., 2008a), increasing considerably their diameter (65-75%) and area (53-65%), compared to the boiled plums (Table 2). Concomitantly with the recover of cell wall diameter and area, candied plums showed an important reduction in intercellular area (34-64% for VV and 70-86% for CA). The recovery of the tissue structure, shown by the microstructure analysis, was also confirmed by the texture analysis of candied plums, which revealed much higher values for the three texture parameters (67-87% for firmness, 56-81% for rigidity, and 66-90% for deformation work), when compared with the boiled plums (Table 3). However, the values were lower than those obtained for the fresh plums, with exception of the deformation work with skin, where values similar to those obtained for the fresh plums were observed. Similar results were obtained for these plums harvested in 2003 (Nunes et al., 2008a).

For both orchards, plums harvested at the 8th harvesting day presented a cell area of candied plums significantly smaller, 16% for VV and 21% for CA, than plums harvested at the 1st day. Firmness, rigidity, and deformation work values were significantly lower for plums from the 8th harvesting day comparing with the 1st harvesting day. As was observed for the boiled plums, these results permit to infer the importance of the ripening stage of the plums to process for the recovering of the cells shape and size after candying and consequently for the texture of candied plums. Plums in a more advanced stage of ripening give rise to a candied product with lower texture quality.

VV candied plums from both harvesting days showed cell diameter and area even higher than the fresh plums, while CA candied plums showed cell diameter and area similar to those obtained for the CA fresh plums (Table 2). VV candied plums presented higher cell diameter and area than CA candied plums. The values of all texture parameters studied were significantly higher for VVc1 comparing to CAc8, except the flesh rigidity. However, no significant differences in the texture parameters of the plums were found between orchards, with and without skin, at the same day of harvesting (Table 3). These results indicated that candied plums showed a recovery of the texture parameters similar

for the same day of harvesting for both orchards, which is in accordance with enzymatic activity and cell wall polysaccharide analysis.

4. Concluding Remarks

Cell wall enzymes activity and changes in cell wall polysaccharides are associated to the changes in the tissue structure and texture of the fruits during ripening, which influence the characteristics of the plums during the thermal and candying process.

Histocytological data (cell diameter, cell area, and intercellular area), texture data (firmness, rigidity, and deformation work), activity of the enzymes pectin methyl esterase, polygalacturonase, and cellulase, and cell wall polysaccharide composition, revealed that CA orchard plums are, probably, at a more advanced stage of ripening than the one that is ascertained by the total soluble solids content and titratable acidity, showing that these conventional established parameters are not enough to, alone, evaluate accurately the stage of ripening of these plums for candying purposes.

Being the ripening stage of primordial importance for the textural characteristics of "Ameixa d'Elvas" candied plums, it is essential to precisely identify the most adequate maturation stage of plums to be processed.

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SEARCH FOR SUITABLE MATURATION PARAMETERS TO DEFINE THE HARVEST MATURITY OF PLUMS (*PRUNUS DOMESTICA* L.). A CASE STUDY OF CANDIED PLUMS.

CAPÍTULO XI

Search for suitable maturation parameters to define the harvest maturity of plums (*Prunus domestica* L.). A case study of candied plums.

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Abstract

Plums (*Prunus domestica* L.) of 'Green Gage' variety, from South-East of Portugal, are used to produce a traditional candied product, "Ameixa d'Elvas", which has a Protected Designation of Origin recognized by the European Union. To obtain a good texture quality in candied plums, it is necessary to define accurate maturation parameters. Parameters such as the total soluble solids (TSS), titratable acidity (TA), TSS/TA, and pH are not always suitable for this purpose. In order to find a more reliable maturation parameter, plums were collected during the commercial harvesting period, in two orchards, Vila Viçosa and Cano in different years (2003 and 2005). Total polysaccharides (PS) and uronic acids (UA) were quantified in the alcohol insoluble residues (AIR) of pulp. In all harvests, the content of polysaccharides and uronic acids present in the AIR increased as the maturity of the fruits progressed. To the dataset that comprised the TSS, TA, TSS/TA, pH, PS, and UA measured in these plums, a linear discriminant classifier was applied to obtain a reliable parameter to predict fruit quality upon candying. The models built showed errors of lack of fitness of 0.005% for the content of UA in the AIR and 0.8% for PS, which contrasted with the errors of 17, 21, 17, and 11%, for the TSS, TA, ratio TSS/TA, and pH, respectively. Considering that the variability associated with the content of PS was higher than that observed in UA estimation, and the easy and fast determination of UA, it is proposed the UA content in AIR be used as a reliable harvesting maturity parameter, complementary to TSS and/or TA, to obtain a high quality candied product. An easy and quick laboratory methodology is proposed for the determination of the UA in plums.

Keywords: 'Green Gage'; ripening; cell wall polysaccharides; pectic polysaccharides; uronic acid; total soluble solids; titratable acidity; pH.

1. Introduction

A large number of physiological, biochemical, and structural changes occur during the ripening of fruits, resulting in modifications identified by measurement of specific physico-chemical parameters. In plums, several maturation parameters have been defined according to characteristics related to color of the skin and flesh, fruit firmness, occurrence of specific volatile compounds, soluble solids content, and of titratable acidity (Bhutani & Joshi, 1995; Abdi, Holford, McGlasson & Mizrahi, 1997; Prasanna, Prabha & Tharanathan, 2007). Skin and flesh color may be useful indicators of ripening but many plum cultivars develop their pigmentation early in growth; therefore, this character has little value for determining harvest date (Bhutani et al., 1995; Abdi et al., 1997). Fruit firmness also has some limitations, since the decrease of firmness is associated with cell enlargement during fruit growth. This characteristic is largely influenced by fruit size, as large fruits show lower firmness than small ones. The production of characteristic volatile compounds is correlated with fruit ripening in many fruits, although in plums no pattern of production was found due to high variability between cultivars (Abdi et al., 1997). Soluble solids content and titratable acidity and their ratio have been suggested as the most reliable maturation parameters for plums, since fruit sweetness gradually increases and acidity decreases during ripening (Bhutani et al., 1995; Prasanna et al., 2007). However, these parameters also have limitations for following maturation, since soluble solids content and titratable acidity have been shown to vary with fruit position on the tree and with environmental conditions (Abdi et al., 1997). For each cultivar, specific maturity parameters need to be defined and, in addition, these parameters need to be adapted depending on the final purpose of the fruit.

Plums (*Prunus domestica* L.) of a special type of ‘Green Gage’ variety, “Rainha Cláudia Verde”, from Alto Alentejo (South-East of Portugal) can be utilised to obtain a traditional candied plum, “Ameixa d’Elvas”, which has a Protected Designation of Origin (PDO) granted by the European Union. To process this variety, the maturity stage at harvest is one of the most important factors for a high texture quality of the final product (Nunes et al., 2008b). For this reason, plums used to produce “Ameixa d’Elvas” are

harvested at a defined maturation point evaluated by their total soluble solids (16 ºBrix) and titratable acidity (1.0 meq malic acid/100 g fruit flesh weight). However, it has been found that plums from an orchard (Cano, CA), although fulfilling these criteria, produced a candied product of low or even no commercial use due to appreciable loss of pulp firmness and skin disruption. However, fruit from another orchard (Vila Viçosa, VV) produced a candied product with good textural properties (Nunes, Santos, Pinto, Lopes-da-Silva, Saraiva & Coimbra, 2008a), when harvested at the same maturation stage as that of CA plums, as determined by total soluble solids and titratable acidity.

The major textural changes resulting in the softening of fruits are due to enzyme-mediated alterations in the composition and structure of cell wall polysaccharides, such as pectic polysaccharides and cellulose, which leads to their partial solubilization (Waldron, Smith, Parr, Ng & Parker, 1997). Cell walls are complex in composition and structure, thus, it is unlikely that any particular enzyme alone is able to significantly modify their properties. A combined action of a number of enzymes acting synergistically is a more plausible cause for the occurrence of the changes needed to make any significant texture changes of fruits (Ali, Chin & Lazan, 2004). The preparation of alcohol insoluble residues (AIR) is a relatively easy and quick method for obtaining plum cell wall polymers (Nunes, Saraiva & Coimbra, 2008c). This method efficiently inactivates enzymes while avoiding significant degradation of cell wall polysaccharides (Coimbra, Delgadillo, Waldron & Selvendran, 1996).

In order to determine if plum cell wall polysaccharides could be used to define more reliable maturity parameters and harvesting fruits for candying, the AIR of plums were prepared from fruits harvested at different dates in two different years and in two different orchards (CA and VV). A linear discriminant classifier was used to relate the maturity characteristics of the fruits to the texture characteristics obtained upon candying. The characteristics assayed to assed maturity were: total soluble solids, titratable acidity, total soluble solids/titratable acidity, pH, total polysaccharides and uronic acids content in the AIR.

2. Materials and Methods

2.1. Plant material and sample preparation

"Rainha Cláudia Verde" is the name given in the PDO to the plums (*Prunus domestica* L.) of 'Green Gage' variety. These plums were collected from two orchards, Vila Viçosa (VV) and Cano (CA), within the PDO region.VV and CA plums were collected in 2003 at five harvest dates: VV plums at 10th, 15th, 18th, 22nd, and 25th of July, and CA plums at 15th, 22nd, 25th, and 30th of July and 1st of August. In 2005, the VV plums were harvested at three harvest dates at 13th, 19th, and 26th of July, and CA plums at two stages ripening at 19th, and 26th of July. The plums were supplied by Fruteco-Fruticultura Integrada, Lda. (Borba, Portugal) and were brought to the laboratory immediately after harvesting.

2.2. Total soluble solids, pH, and titratable acidity

The plums' juice was obtained by squeezing the plum flesh followed by filtration through a glass fibre filter (Whatman GF/C). Total soluble solids, pH, and titratable acidity were determined on the filtrate. Total soluble solids (ºBrix) were determined by measuring the refractive index of the juice with a hand held refractometer (ATC-1E, Atago Co. Lda., Japan). Titratable acidity was measured by titration with 0.1 M NaOH to an endpoint of pH 8.1 using an automatic pH–stat (Crison micro TT2022, Alella, Spain) using 6 g of juice diluted with 50 mL of distilled water. Titratable acidity was calculated and expressed as meq of malic acid/100 g of deseeded fruit.

2.3. Preparation of cell wall material

Plums were deseeded and peeled and the flesh was dispersed in ethanol using a proportion of 4 mL to 1 g of solids. The suspension was boiled 10 min. The mixture was

cooled and filtered through a glass fibre filter (Whatman GF/C). The residue was dispersed again in 85% ethanol, boiled for 10 min and filtered. The residue was then washed with diethyl ether and allowed to dry at room temperature. The dried material was considered to be the alcohol insoluble residue (AIR).

2.4. Carbohydrate analysis

Monosaccharides were released from cell wall polysaccharides by a pre-hydrolysis in 0.2 mL of 11 M H_2SO_4 for 3 h at room temperature followed by 2.5 h hydrolysis in 1 M H_2SO_4 at 100 ºC (Selvendran, March & Ring, 1979). Neutral sugars were analyzed after conversion to their alditol acetates by GC, using 2-deoxyglucose as internal standard (Coimbra et al., 1996) and GC analysis as described by Nunes et al. (Nunes, Rocha, Saraiva & Coimbra, 2006). Cellulosic glucose was calculated as the difference between the content found with and without 11 M H_2SO_4 pre-hydrolysis.

Uronic acids (UA) were quantified by a modification (Coimbra et al., 1996) of the 3-phenylphenol colorimetric method (Blumenkrantz & Asboe-Hansen, 1973). Samples were prepared by hydrolysis in 0.2 mL of 11M H_2SO_4 for 3 h at room temperature followed by 1 h in 1 M H_2SO_4 at 100 ºC. A calibration curve based on D-galacturonic acid as standard was used to calculate UA concentration.

2.5. Statistical analysis

Quantitative analyses are presented as mean values and the reproducibility of the results is expressed as standard deviation. Statistical analysis of the experimental results was carried out based on Student's *t* test. Significant differences were considered at the level of $p<0.05$.

2.6. Linear discriminant classifier

A linear discriminant classifier (Vandeginste, Massart, Buydens, De Jong, Lewi & Smeyers-Verbeke, 1998) was applied to assess the classification ratios of total soluble solids, titratable acidity, total soluble solids / titratable acidity, pH, total polysaccharides, and uronic acids. This classifier was based on least squares regression by estimating the slope and the intercept, defining a line boundary that can be used to classify samples. The values of the above parameters (summarized in Table 1) were used to build the classifier. A dummy vector was filled with 0 (zeros) and 1 (ones) for the cases of poor and good final product, respectively. A Monte-Carlo model validation in tandem with the linear discriminant classifier was used to compute the classification ratios of each parameter of interest, two thirds of the samples were used for calibration purposes and one third was used for validation. Each Monte-Carlo model was run 1,000 times. From these 1,000 models for each parameter the averaged linear classifier parameters (slope and intercept) and models lack of fitness (LOF) were calculated. In addition, and for each parameter, a random permutation test was performed.

3. Results and Discussion

3.1. Characterization of plum samples

The dataset used in this study was produced from fruit collected from two orchards (VV and CA), in two years (2003 and 2005), harvested at different maturities (Table 1). The content of total soluble solids (TSS) varied from 12.8 ºBrix in CA harvested on 15th July 2003 to 21.9 ºBrix in CA harvested on 1st August 2003: this parameter increased as the fruit from all locations and years became more mature. Titratable acidity (TA) varied from 0.88 meq of malic acid in CA harvested on 1st August, 2003 to 1.18 in CA harvested on 19th July 2005, and decreased as the fruit matured. TSS/TA ranged from 11.0 ºBrix/meq of malic acid in CA harvested on 15th July 2003 to 24.9 CA harvested on 1st August 2003, and this parameter increased with fruit maturity in all four ripening sets. The pH of the

fruits' juice ranged from 3.16 for CA plums harvested on 19th July 2005 to 3.49 for CA plums harvested on 1st August 2003 and increased as the fruit ripened.

Table 1. Total soluble solids (TSS), titratable acidity (TA), TSS/TA, and pH of the fruits and total polysaccharides (PS) and uronic acids (UA) of AIR from plums from Vila Viçosa (VV) and Cano (CA).

Harvesting Day	*TSS (ºBrix)* (n=3)	*TA (meq of malic acid/100 g)* (n=3)	*TSS/TA (ºBrix/meq of malic acid)* (n=3)	*pH* (n=2)	*PS (mg/g AIR)* (n=4)	*UA (mg/g AIR)* (n=4)
VV 2003						
10 Jul 03	14.8 ± 0.1^{a}	1.12 ± 0.02^{a}	13.3 ± 0.1^{a}	3.21	534 ± 21^{a}	239 ± 18^{a}
15 Jul 03	15.2 ± 0.2^{a}	1.02 ± 0.01^{b}	14.8 ± 0.2^{b}	3.19	598 ± 32^{b}	197 ± 14^{a}
18 Jul 03	16.6 ± 0.1^{b}	1.00 ± 0.01^{b}	16.6 ± 0.2^{c}	3.26	781 ± 25^{c}	386 ± 14^{b}
22 Jul 03	18.8 ± 0.2^{c}	0.94 ± 0.01^{c}	20.0 ± 0.3^{c}	3.29	825 ± 19^{c}	425 ± 17^{c}
25 Jul 03	21.2 ± 0.1^{d}	0.90 ± 0.01^{d}	23.7 ± 0.1^{d}	3.30	906 ± 14^{d}	453 ± 12^{c}
VV 2005						
13 Jul 05	14.5 ± 0.1^{a}	1.14 ± 0.03^{a}	12.7 ± 0.2^{e}	3.19	493 ± 12^{a}	190 ± 5^{a}
19 Jul 05	16.1 ± 0.1^{e}	1.03 ± 0.01^{b}	15.7 ± 0.3^{f}	3.22	623 ± 25^{b}	260 ± 21^{a}
26 Jul 05	19.6 ± 0.1^{f}	0.90 ± 0.01^{d}	21.7 ± 0.1^{g}	3.32	756 ± 38^{c}	359 ± 35^{b}
CA 2003						
15 Jul 03	12.8 ± 0.1^{g}	1.16 ± 0.01^{a}	11.0 ± 0.2^{h}	3.22	644 ± 28^{b}	293 ± 78^{a}
22 Jul 03	15.6 ± 0.1^{a}	1.10 ± 0.01^{a}	14.2 ± 0.0^{b}	3.28	747 ± 29^{c}	$416 \pm 26^{b,c}$
25 Jul 03	19.7 ± 0.1^{f}	1.02 ± 0.01^{b}	19.3 ± 0.2^{c}	3.34	823 ± 14^{c}	432 ± 14^{c}
30 Jul 03	21.5 ± 0.2^{d}	$0.92 \pm 0.01^{c,d}$	23.3 ± 0.1^{d}	3.38	834 ± 11^{c}	472 ± 82^{c}
1 Aug 03	21.9 ± 0.1^{d}	0.88 ± 0.01^{d}	24.9 ± 0.3^{i}	3.49	917 ± 24^{d}	486 ± 21^{c}
CA 2005						
19 Jul 05	13.7 ± 0.1^{h}	1.18 ± 0.01^{a}	11.6 ± 0.1^{h}	3.16	690 ± 18^{e}	255 ± 20^{a}
26 Jul 05	15.7 ± 0.2^{a}	1.04 ± 0.01^{b}	15.1 ± 0.2^{b}	3.27	784 ± 27^{c}	357 ± 25^{b}

Mean ± standard deviation. Within columns, means with different superscript letters are significantly different ($p<0.05$).

The plums used to produce candied "Ameixa d'Elvas" are commonly harvested when the TSS is approximately at 16 ºBrix and the TA is higher than 1.0 meq of malic acid. According to these criteria, the plums from VV orchard collected until 15th July 2003 and 19th July 2005, and the plums from CA orchard collected until 22nd July 2003 and 26th July 2005 should produce good quality candied fruits. Figure 1a shows the TSS of the plums from the two orchards. For VV, all samples with a TSS higher than 16.6 were unsuitable for processing; hence, this criterion of maturity was applicable to this orchard. However, this criterion seems not to be applicable to the plums from CA orchard. In fact, the CA plums collected on 22nd July 2003 and 26th July 2005, although having values of TSS suitable to classify them as adequate to be candied, produced poor final products with low pulp consistency and appreciable skin disruption (Nunes et al., 2008a; Nunes et al., 2008b).

TA is also not a reliable predictor of processing quality as the plums collected from CA on 22nd and 25th July 2003 and 26th July 2005, although having values of TA suitable to classify them as adequate to be candied, produced poor texture quality products. TSS/TA was also unsuitable as a criterion to evaluate the plums from CA (Figure 2a). In contrast to TSS and TA and their ratio, the measurement of juice pH allows a separation of fruit that give good products from those that do not (Figure 2b). However, the pH range separation of good from poor quality fruit is too small to obtain an unequivocal definition of the harvesting day for candying based on this parameter. As these parameters seem not to be reliable enough to evaluate the maturity stage of plums as well as to determine precisely the moment to harvest fruits for processing, other parameters, based on cell wall polysaccharide composition of the fruits, were assessed.

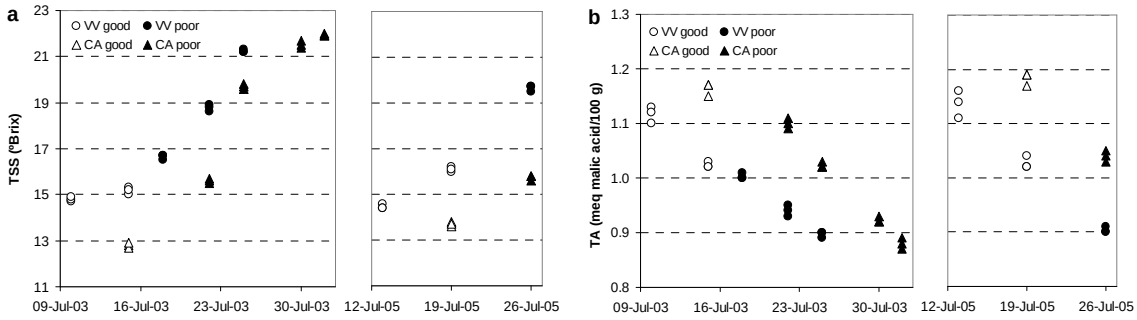

Figure 1. (a) Total soluble solids (TSS) and (b) titratable acidity (TA) of plums from Vila Viçosa (VV) and Cano (CA) orchards in 2003 and 2005.

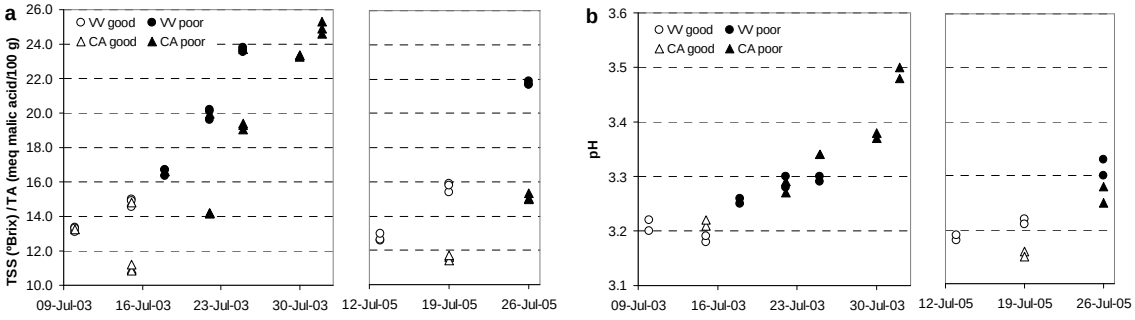

Figure 2. (a) Ratio of total soluble solids and titratable acidity (TSS/TA) and (b) pH of plums from Vila Viçosa (VV) and Cano (CA) orchards in 2003 and 2005.

3.2. Total polysaccharides and uronic acids content in AIR

The total polysaccharides (PS) present in the AIR varied from 493 mg/g AIR in VV plums harvested on 13th July 2005 to 917 mg/g AIR in CA plums harvested on 1st August 2003 (Table 1). This parameter increased as the maturity of the fruit progressed in all four ripening sets studied. The uronic acids (UA) present in the AIR, which represent the pectic polysaccharides of the fruit, varied from 190 mg/g AIR in VV harvested on 13th July 2005 to 486 mg/g AIR in CA harvested on 1st August 2003. As observed for the PS, the content in UA increased as the maturity of the fruit proceeded in all four ripening sets studied.

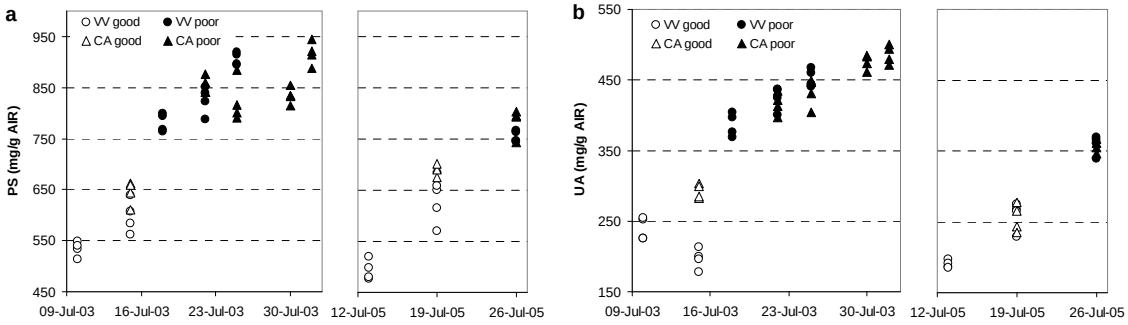

Figure 3. (a) Total polysaccharides (PS) and (b) uronic acids (UA) content in AIR of plums from Vila Viçosa (VV) and Cano (CA) orchards in 2003 and 2005.

Figure 3 shows the content of PS (Figure 3a) and UA (Figure 3b) in the AIR of plums from the two orchards. A visible separation is obtained between the samples that give good or poor texture quality product for both parameters. Cell wall polysaccharide composition seems to provide the information about the stage of ripening of the fruit suitable to predict its texture behavior upon candying.

3.3. Linear discriminant classifier

In order to assess the classification ratios of TSS (3 replicates of 15 fruit samplings), TA (3 replicates of 15 fruit samplings), TSS/TA (3 replicates of 15 fruit samplings), pH (2 replicates of 15 fruit samplings), PS (4 replicates of 15 fruit samplings), and UA (4 replicates of 15 fruit samplings) for the determination of a reliable classifier for the

prediction of the ideal ripening stage of plums for candying, a linear discriminant classifier was applied. The results obtained are shown in Table 2.

Table 2. Linear discriminant classifier results as a function of the total soluble solids (TSS), titratable acidity (TA), TSS/TA, and pH of the fruit and total polysaccharides (PS) and uronic acids (UA) of AIR.

Parameter	*Number of samples*	*Slope*	*Intercept*	*LOF (%)*	*Permutation (LOF, %)*
TSS (ºBrix)	45	-0.127 ± 0.008	2.58 ± 0.16	17	~ 59
TA (meq malic acid)	45	3.55 ± 0.20	-3.23 ± 0.20	21	~ 59
TSS/TA	45	-0.08 ± 0.01	1.78 ± 0.20	17	~59
pH	30	-4.47 ± 0.86	15.0 ± 2.8	11	~ 57
PS (mg/g AIR)	60	-0.0033 ± 0.0034	2.9 ± 0.1	0.8	~ 49
UA (mg/g AIR)	60	-0.0045 ± 0.0001	1.95 ± 0.06	0.005	~ 49

Mean ± standard deviation.

Using the slope and the intercept for each parameter, it is possible to predict the texture of the fruits upon candying. A value near one means that the fruit is suitable for candying according to this parameter and, on the contrary, a value near zero means that the fruit is not suitable. When the TSS is used as a maturity parameter to select the fruits for candying, an error (LOF) of 17% is obtained. Also, an error of the same order of magnitude is obtained for TA (21%) and TSS/TA (17%), whereas the error given by the pH of the fruit's juice is 11%. These high values confirm that plums to be candied. Nevertheless, the errors obtained were relatively lower when the PS and the UA content in AIR of plums were used as maturity parameters to select the fruits for candying (0.8% for PS and 0.005% for UA content). Considering the high standard error in the slope for PS, the content UA in the AIR seems the parameter of choice. In order to assess if the models proposed were not due to chance, permutation tests were performed. This is based on the principle that a random permutation of the samples grouping should give 50% of correct answers and 50% of incorrect ones due to the arbitrary classification of the samples into 0 or 1. The permutation test performed confirmed that the content of UA in the AIR is a reliable parameter to predict the final quality of the candied plums, since the lack of fitness obtained when this test was performed it was near 50%.

4. Conclusion

This work shows that the estimation of the total amount of uronic acids in the AIR of the plums is a reliable maturation parameter able to define the harvesting point of "Rainha Cláudia Verde" plums for candying. This methodology, which is based on a colorimetric method, can be performed in an easy and quick way within a laboratory and provides an accurate assessment of harvest date for candying than is provided by total solids or titratable acidity. Total solids and titratable acidity are reliable parameters to follow the maturation of the plum with little extra time or effort. However, for the definition of the harvesting point for candying, complementary data from UA is required. The preparation of the AIR by boiling, in a test tube, of 0.5 g of plums flesh in 2 mL of ethanol, filtration, wash with diethyl ether, and drying, can be performed in less than 30 min. From it, UA can be released by 1 h in 1 M H_2SO_4 at 100 ºC, and colorimetrically quantified in the following 30 min. The time required for the analysis of a single sample is not very different if a range of 10-12 samples are used simultaneously. This gives a maximum time of laboratorial analysis of 2 h, which is a feasible time for the maturity control of the fruits of the orchard.

Abbreviations Used

AIR, alcohol insoluble residue; CA, Cano; LOF, lack of fitness; PDO, protected designation of origin; PS, polysaccharides; TA, titratable acidity; TSS, total soluble solids; TSS/TA, total soluble solids/titratable acidity; UA, uronic acids; VV, Vila Viçosa.

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CAPÍTULO XII

CONCLUSÃO

Neste trabalho foi proposta e optimizada uma nova metodologia para a determinação simultânea do grau de esterificação e acetilação de amostras de polissacarídeos das paredes celulares. A metodologia consiste na extracção do metanol e do ácido acético presente na fase de vapor (HS) da amostra após a saponificação dos polissacarídeos, utilizando a técnica de microextracção em fase sólida (SPME). Os compostos extraídos são posteriormente separados por cromatografia em fase gasosa (GC) e detectados utilizando um detector de ionização de chama (FID). As condições experimentais de extracção do metanol e do ácido acético foram optimizadas, em que a fibra de SPME com o revestimento DVB/Carboxen/PDMS (Divinilbenzeno/Carboxen/Polidimetilsiloxano) durante 30 minutos revelou ser o melhor procedimento para a extracção. Os compostos são quantificados usando curvas de calibração externas. Uma relação linear entre a concentração do composto e a sua área cromatográfica foi obtida para concentrações que variavam entre 40 e 100 mg/L para o metanol e entre 25 e 105 mg/L para o ácido acético, em que não foi verificada interferência da presença de um composto na quantificação do outro. Esta proposta de metodologia (HS-SPME-GC-FID) foi usada para analisar diversas amostras de polissacarídeos pécticos e extractos de parede celular com diferentes graus de esterificação (40-85%) e acetilação (5-20%). Os resultados obtidos são comparáveis aos valores determinados por injecção directa no GC das amostras contendo metanol e ácido acético. A metodologia de HS-SPME-GC-FID demonstrou poder ser utilizada para a análise dos polissacarídeos das paredes celulares de plantas independentemente da sua origem e tipo. Esta metodologia revelou ser mais simples, rápida, limpa e reprodutível na determinação do conteúdo em metanol e ácido acético das amostras.

A desesterificação dos polissacarídeos pécticos da parede celular ocorre devido à acção de uma enzima específica, a pectina metilesterase (PME). Devido à sua importância para a textura dos frutos frescos e processados e ao reduzido conhecimento sobre as suas características nas ameixas, a PME foi purificada e caracterizada na Ameixa d'Elvas em fresco. A enzima purificada por cromatografia de afinidade foi também caracterizada a nível bioquímico. A PME da Ameixa d'Elvas possui uma massa molecular de 31 kDa e é constituída por duas isoenzimas com pontos isoeléctricos neutros (6,8 e 7,0), tendo uma

actividade máxima a 65 ºC e pH 7,5. A estabilidade térmica da enzima purificada é superior a pH 7,5 em relação ao pH 4, o valor de pH idêntico ao das ameixas em fresco. Os estudos de inactivação térmica demonstraram que a enzima é constituída por uma fracção termolábil e outra termoestável, pois a inactivação teve um comportamento bifásico. A PME da ameixa revelou ser relativamente estável a tratamentos térmicos (5 minutos a 50-60 ºC) e de alta pressão (≤ 600 MPa), em comparação com PMEs purificadas de outros frutos. Estes resultados mostram que a actividade de PME deve ser considerada durante o processamento das ameixas.

A composição em compostos fenólicos da Ameixa d'Elvas não processadas foi estudada, tendo-se analisado ameixas produzidas nos dois pomares da região DOP (Vila Viçosa e Cano). Os resultados obtidos foram comparados com ameixas da mesma variedade de origem geográfica diferente e com ameixas de outras variedades e espécie. Os compostos fenólicos foram identificados e quantificados na polpa e na pele das ameixas por cromatografia de elevada pressão de fase reversa (RP-HPLC) após a tiólise dos extractos metanólicos. A polpa das ameixas é constituída por duas classes de compostos fenólicos, os ácidos hidroxicinâmicos e os flavanóis, enquanto que a pele contém mais uma classe de compostos, os flavonóis. O uso da tiólise permitiu demonstrar que os principais compostos fenólicos presentes na polpa das ameixas são as procianidinas, contrariamente ao que está descrito na literatura em que os ácidos hidroxicinâmicos era a principal classe de compostos fenólicos presentes.

As ameixas da variedade 'Rainha Cláudia Verde' revelaram ter uma concentração superior em compostos fenólicos em relação às outras duas variedades de ameixa estudadas e a outras ameixas descritas na literatura. As Ameixas d'Elvas possuem ainda quantidades superiores, na polpa e na pele, em relação às ameixas da mesma variedade mas produzidas noutra área geográfica. Estas ameixas DOP são também constituídas por uma elevada concentração em procianidinas e com maior grau de polimerização. Entre as Ameixas d'Elvas produzidas nos dois pomares da região DOP não se verificaram diferenças significativas em quantidade nem tipo de compostos identificados. Estes resultados, apesar de preliminares, parecem indicar que as Ameixas d'Elvas têm uma composição em compostos fenólicos da polpa e/ou da pele que as poderá distinguir de

outras variedades e espécies de ameixas e/ou de ameixas da mesma variedade produzidas fora da região delimitada pelo DOP.

Com o objectivo de conhecer a composição volátil da Ameixa d'Elvas confitada e de tentar estimar quais os compostos voláteis responsáveis pelo seu aroma peculiar, duas técnicas de extracção de compostos voláteis foram utilizadas, a extracção e destilação simultânea (SDE) e a microextracção em fase sólida (SPME). A combinação destas técnicas permitiu primeiro analisar a composição volátil e identificar os compostos voláteis que estavam presentes na Ameixa d'Elvas confitada em concentrações superiores ao seu limite de percepção sensorial e destes os que estavam presentes na fase de vapor da ameixa confitada e que provavelmente eram responsáveis pelo aroma do produto.

A composição volátil da Ameixa d'Elvas confitada reflecte a complexidade de reacções que ocorreram na maturação do fruto e aquelas que aconteceram devido ao processo de confitagem (tratamento térmico e elevada concentração de açúcar). A Ameixa d'Elvas confitada é constituída por compostos voláteis característicos do fruto não processado, como os ácidos, terpenóides, lactonas e ésteres, por compostos voláteis produzidos durante o tratamento térmico, como os furanos e alguns compostos carbonílicos, e por compostos voláteis que indiciam a ocorrência de fermentação, tais como o etanol, o eugenol e os ésteres etílicos. De todos os compostos identificados, 19 estão presentes em concentrações superiores ao seu limite de percepção sensorial e desses, 11 foram detectados na fase de vapor da amostra: octanoato de etilo, nonanal, eugenol, acetato de 2-feniletilo, linalol, benzoato de etilo, benzaldeído, 2-heptenal, ácido hexadecanóico, ácido 3-metilbutanóico e β-citronelol. A maioria destes compostos estão descritos na literatura como tendo odores doces e frutados. A presença de alguns destes compostos voláteis, como por exemplo o linalol e o β-citronelol, que nunca foram descritos na literatura para ameixas processadas termicamente em presença de sacarose, podem ser responsáveis pelo aroma característico da Ameixa d'Elvas confitada.

A calda de açúcar da Ameixa d'Elvas, usada para processar e conservar este produto, tem também um importante contributo para o seu aroma. Todos os compostos, excepto o 2-heptenal, identificados como responsáveis pelo aroma dos frutos confitados estão também presentes na fase de vapor da calda de açúcar. A presença destes compostos na calda de

açúcar demonstrou que existe a transferência de compostos do fruto para a calda durante o processamento e armazenamento.

Esta tese de Doutoramento permitiu conhecer as características físico-químicas da Ameixa d'Elvas e as suas alterações com o processo de confitagem. As características estudadas foram a estrutura e organização dos tecidos do parênquima, a textura do fruto e os polissacarídeos da parede celular. Neste estudo foram usadas ameixas não processadas, cozidas e confitadas, provenientes dos dois pomares, Vila Viçosa e Cano. As ameixas foram colhidas com o mesmo grau de maturação avaliado pelos parâmetros convencionais (sólidos solúveis totais e acidez titulável) e segundo o que é utilizado pela empresa para seleccionar os frutos para confitar (16 – 17 ºBrix e 1 meq de ácido málico por 100 g de fruto fresco).

A estrutura e organização celular foram observadas por microscopia electrónica de varrimento (SEM) ao longo do processo de confitagem. No geral, as ameixas não processadas possuem células do parênquima isodiamétricas com uma área entre 4 e 6 μm^2 com uma organização celular regular e espaços intercelulares reduzidos (0.02-0.05 μm^2), em que alguns feixes vasculares aparecem dispersos nos tecidos do parênquima da ameixa. A textura dos frutos foi avaliada por três parâmetros diferentes, a firmeza, a rigidez e o trabalho necessário para a deformação dos tecidos, determinados por testes de perfuração em ameixas com pele e sem pele. As ameixas não processadas são frutos relativamente rígidos, principalmente quando analisadas com pele. O tratamento térmico origina a diminuição da adesão intercelular com degradação da lamela média, a irregularidade da forma das células e a ruptura da estrutura dos feixes vasculares. A degradação da microestrutura promove a diminuição drástica da firmeza (80-94%), rigidez (93-98%) e do trabalho necessário para a deformação dos tecidos (72-91%). A imersão das ameixas cozidas na calda de açúcar durante longos períodos de tempo (mínimo de 2 meses) origina uma recuperação da forma e tamanho das células do parênquima e da adesão intercelular nos tecidos da polpa das ameixas confitadas. Esta recuperação da estrutura celular reflecte-se também num aumento da firmeza e do trabalho de deformação das ameixas para valores próximos aos da polpa da ameixa não processada. A análise da textura e microestrutura revelou que a recuperação da textura nas ameixas confitadas está relacionada com a

estrutura das células do parênquima, nomeadamente com a recuperação ao nível da lamela média.

O estudo dos polissacarídeos da parede celular foi realizado nas mesmas ameixas onde foram analisadas a textura e microestrutura. A extracção sequencial dos polissacarídeos da parede celular revelou que a polpa das ameixas não processadas é constituída principalmente por polissacarídeos pécticos (62%) e celulose (15%). O tratamento térmico origina a sua degradação e solubilização, o que pode explicar a alteração da estrutura das células do parênquima, principalmente a perda da adesão celular, e a perda de textura dos frutos. Elevadas quantidades de polissacarídeos pécticos solúveis em água com um elevado grau de esterificação (83%) são detectadas nas ameixas após o tratamento térmico, tendo estes polímeros a característica de poderem formar géis na presença de elevadas concentrações de açúcar. A desidratação osmótica e a difusão de sacarose para o interior dos frutos que ocorre durante a confitagem promovem a interacção destes polissacarídeos pécticos, justificando o aumento da textura dos frutos e a recuperação da estrutura das células com a confitagem. Esta recuperação ocorre devido à formação de um gel no interior dos frutos, principalmente na região da lamela média, num processo semelhante ao que se observa nas compotas dos frutos. O conteúdo em polissacarídeos foi também determinado na calda de açúcar onde as ameixas foram processadas e armazenadas, sendo principalmente constituída por polissacarídeos pécticos com elevado grau de esterificação (68%). Estes polissacarídeos, como são muito solúveis, difundiram facilmente dos frutos para a calda de açúcar durante a confitagem, contribuindo para o aumento da sua viscosidade.

Apenas após o processamento são encontradas diferenças significativas a nível da textura entre as ameixas provenientes dos dois pomares. As ameixas do Cano têm valores inferiores para as ameixas fervidas e confitadas, confirmando a sua qualidade inferior. A recuperação da textura com a confitagem revelou ser limitada pela extensão da degradação dos tecidos durante o tratamento térmico a que as ameixas foram sujeitas. Nas ameixas do pomar do Cano existe uma maior degradação dos tecidos e, consequentemente, uma menor recuperação da textura e da estrutura celular com a confitagem. Estas observações a nível de microestrutura e textura ao longo do processo de confitagem foram também confirmadas pela análise dos polissacarídeos das paredes celulares. As ameixas do Cano, após o tratamento térmico, apresentam uma maior degradação dos polissacarídeos da

parede celular, enquanto que nas ameixas de Vila Viçosa essa degradação foi apenas observada após o processo de confitagem. Estes resultados demonstram que as ameixas do Cano são mais susceptíveis ao tratamento térmico. Este facto pode ser explicado pela maior actividade das enzimas da parede celular, poligalacturonase (PG) e celulase (Cel), nas ameixas não processadas do Cano em relação às de Vila Viçosa. Estas enzimas são responsáveis pela diminuição da integridade da parede celular e, consequentemente, da firmeza dos tecidos. Apesar de não terem sido detectadas diferenças significativas na análise da textura dos frutos não processados entre os dois pomares, as ameixas do Cano apresentam uma maior solubilização dos polissacarídeos da parede celular e esta diferença influencia o comportamento das ameixas durante o processo de confitagem.

As diferenças encontradas nos polissacarídeos da parede celular e na actividade das enzimas entre as ameixas dos dois pomares parecem estar relacionadas com diferentes estados de maturação, apesar de possuírem o mesmo conteúdo em sólidos solúveis totais e a mesma acidez titulável. As ameixas do Cano pareciam estar num estado de maturação mais avançado. Para confirmar esta hipótese, a microestrutura e a textura foram estudadas ao longo do processamento para ameixas colhidas em dois estados de maturação, quer em Vila Viçosa, quer no Cano. Os frutos foram colhidos no primeiro dia da colheita para confitar das ameixas de Vila Viçosa e no dia de colheita das ameixas do Cano, de acordo com o conteúdo em sólidos solúveis totais e a acidez titulável. Este estudo confirmou que a maturação das ameixas influencia a microestrutura e textura dos frutos ao longo do processo de confitagem, pois um estado de maturação mais adiantado origina uma maior degradação da estrutura celular e da textura com o tratamento térmico e uma menor capacidade de recuperação com a confitagem. Os frutos colhidos no mesmo dia nos dois pomares apresentaram parâmetros de textura semelhantes após a confitagem. A actividade das enzimas PME, PG e Cel foi também quantificada ao longo da maturação das ameixas, demonstrando que a actividade de PG e Cel aumenta com a maturação dos frutos, enquanto que a PME aumenta na primeira fase e depois diminui. Para o mesmo dia de colheita os frutos de ambos os pomares têm actividades enzimáticas e composição em polissacarídeos da parede celular idênticas. Este estudo confirmou que as ameixas do Cano estavam num estado de maturação mais adiantado, a nível dos polissacarídeos e das enzimas da parede celular, em comparação com as de Vila Viçosa, quando foram colhidas para serem processadas, explicando as diferenças de textura das ameixas confitadas.

Este trabalho comprovou que a actividade das enzimas da parede celular e as modificações que ocorrem nos polissacarídeos estão relacionados com as modificações da estrutura celular dos tecidos e da textura dos frutos com a sua maturação, influenciando as características das ameixas com o processo de confitagem.

Os parâmetros convencionais usados para avaliar a maturação de frutos (sólidos solúveis totais e acidez titulável) mostraram não ser adequados para seleccionar as ameixas para confitar. Com o objectivo de verificar se os polissacarídeos das paredes celulares poderiam ser usados como parâmetros para avaliar o estado de maturação das ameixas, o material polimérico da polpa das ameixas foi obtido por preparação do resíduo insolúvel em álcool (AIR) e a sua composição em polissacarídeos foi determinada. O conteúdo em polissacarídeos totais e ácidos urónicos aumenta gradualmente ao longo da maturação das ameixas provenientes dos dois pomares. Uma concordância com a obtenção de um bom produto confitado foi também observada, em que ameixas dos dois pomares com conteúdos inferiores de polissacarídeos e ácidos urónicos originam um produto confitado com uma boa textura. Esta tendência foi observada em ameixas colhidas em dois anos, 2003 e 2005. Uma análise linear discriminante foi aplicada para todos os parâmetros analisados nas amostras (sólidos solúveis totais, acidez titulável, pH, polissacarídeos totais e ácidos urónicos) para determinar o melhor parâmetro de maturação. Os modelos matemáticos comprovaram que o conteúdo em polissacarídeos totais e ácido urónico no AIR conseguem prever o comportamento das ameixas após a confitagem, em termos de textura. No entanto, o conteúdo em ácidos urónicos revelou ser o melhor parâmetro para seleccionar as ameixas para o processo de confitagem durante a sua maturação, pois tem uma percentagem de erro muito reduzida em comparação com os outros parâmetros.

Em conclusão, este estudo revelou que as ameixas do Cano geralmente originam ameixas confitadas com má qualidade a nível da textura por estarem num estado de maturação muito avançado que não consegue ser avaliado pelos parâmetros de maturação normalmente usados pela indústria. O estado de maturação das ameixas para confitar deve ser avaliado pelo conteúdo em ácido urónico no AIR, pelo que se propõe esta metodologia rápida e fiável que permite determinar a altura ideal para colher as ameixas para confitar em todos os pomares da região DOP.